Exploring the use of Microbial Fuel Cells for the online detection of organic micropollutants in water

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ABSTRACT

This work explores the possibilities for the use of Microbial Fuel Cells (MFCs) as biosensors for the detection of biologically active organic micropollutants in water streams. The work centred on the detection of the molecules bisphenol-a (BPA), 17β-estradiol (estradiol), naproxen and diclofenac, which are contained within a European Union "watch-list" of emerging micropollutants. This lists them as chemicals of concern which require monitoring due to their potential effects of the environment and on human health. As such there is a pressing need for detection methods for effective monitoring.

All four micropollutants were detected at concentrations at or near the range found in wastewaters. Triclosan was detected at 0.10 μg mL^{-1}, diclofenac at 0.05 μg mL^{-1}, bisphenol-a at 0.1 μg mL^{-1}, and estradiol at 0.5 μg mL^{-1}.

The study also investigated the effects of long-term exposure to these toxicants, which seem to have an effect on the performance of MFCs over time, although this effect is not yet fully understood or quantified.

It was noted that control of anode potential (and therefore chemical overpotential) was particularly effective in stabilising the baseline current of the MFC-based biosensors, and dramatically improved repeatability of results. As such it is a promising way forward for the control of MFC-based biosensors.

This work also investigated the simultaneous detection and removal of these micropollutants and for the first time, reports that these micropollutants can be almost completely removed using a cascade of MFCs, whereby they are fed through several MFCs in succession, although some of the removal is attributed to absorption processes rather than metabolism of the substrate.

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LIST OF ABBREVIATIONS

A	ampere
AAS	Atomic Absorption Spectroscopy
AES	Atomic Emission Spectroscopy
BOD	Biochemical Oxygen Demand
BPA	Bisphenol-A
BVM	Butler-Volmer-Monod Equation
cm	centimetre
COD	Chemical Oxygen Demand
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic Acid
e-	Electron
EIS	Electrical Impedance Spectroscopy
EU	European Union
GC	Gas Chromatography
H+	Proton
km	kilometre
L	litre
LC-MS	Liquid Chromatography - Mass Spectrometry
m	metre
mA	milliampere
mA	microampere
MFC	Microbial Fuel Cell
mg	microgram
mg	milligram
min	minutes
mL	millilitre
mm	millimetre
MS	Mass Spectrometry
mV	millivolt
NSAID	Non-steroidal Anti-Inflammatory Drug
PEM	Proton Exchange Membrane
s	seconds
SCE	Standard Calomel Electrode
SDS	Sodium dodecyl sulfate
SHE	Standard Hydrogen Electrode
TOC	Total Organic Carbon
V	Volt
W	Watts
Ω	Ohm
(w/w)	Weight per Weight

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1.0 – INTRODUCTION

As global population increases, the demand for safe, clean water for drinking and agriculture also increases. At this time of unprecedentedly high global population, there is a scarcity of freshwater available, particularly in developing regions of the world. It is therefore important to be able to treat and recycle water effectively. Technologies which can rapidly monitor water quality are important to help ensure that water treatment is effective, by observing the level of toxicants which occur in partially or completely treated wastewater. [1-5]

The use of biologically active molecules, such as pharmaceuticals, pesticides, antifungal agents, etc. is increasing across the globe. As such they are often found in wastewater, even after treatment at low levels, and have caused concern as their effect on ecosystems and on humans is difficult to quantify. As such, many have been classed as emerging pollutants and are the subject of studies by bodies such as the EU.[6]

They are biologically active molecules, which can have different effects on different organisms. In parts of Asia, diclofenac (a common painkiller) has found to be responsible for a decline in vulture populations, as the drug is commonly administered to cattle, which were later being eaten by vultures, which are affected by this drug differently to cattle and humans.[7, 8]

Although the concentration of individual toxicants in water may be low (micropollutant: pictograms per litre to nanograms per litre), the effect of the mixing and interaction of several together (co-contamination) is of high concern due to potential effects on human and aquatic life.[9]

Examples of such biologically active micropollutants and their effects can be found in Table 1.

Table 1 - A Range of Micropollutants Common in Wastewater (adapted)[9]

Origin/Usage	Class	Selected Examples	Associated Problems
Industrial Chemicals	Solvents	Tetrachloromethane	Drinking water contamination
	Intermediates	Methyl-*t*-butylether	
	Petrochemicals	Benzene, toluene, xylene	
Industrial Products	Additives	Phthalates, Bisphenol-A	Biomagnification, long-range transport, endocrine disruption
	Lubricants	Bisphenols	
	Flame Retardants	Polybrominates	
Consumer Products	Antibiotics	Tetracycline, Sulfamethoxizole, Triclosan	Bacterial resistance, non-target effects
	Hormones	Estradiols	Feminisation of fish
	Pain-killers	Diclofenac, Naproxen, Paracetamol	Weakening of bird eggshells, non-target effects
	Personal Care Products	UV-blockers	Numerous, unknown
Biocides	Pesticides	DDT, Atrazine	Toxic effects, persistent metabolites, effects on primary producers (*e.g.* algae)
	Nonagricultural Biocides	Triclosan, Tributyltin	Non-target effects, persistent metabolites, endocrine effects
Transformation Products	Metabolites from all the above		Bioaccumulation, drinking water quality effects, multiple unknown hazards

Microbial Fuel Cells (MFCs) have shown great potential to be used as biosensors for water quality.[2, 10-12] MFCs are devices which, through the action of specific microbes (electrogens) generate an electrical current *via* the metabolisation of organic compounds (fuel) by transferring electrons generated in this process to an anode. As a result, the current generated by MFCs is directly proportional to the rate of metabolisation of the fuel by the electrogens.

Where the MFC operates under saturated fuel concentrations, provided other variables are controlled (*e.g.* temperature, anode potential etc.) any alteration in the output current can

be attributed to the presence of toxicants in the system.[13] The presence of this toxicant has been shown to affect the metabolism of the bacteria, as well as their growth rate.[1] The MFC can therefore act as an indicator for biologically active compounds in water.[14] As such, they have been used to monitor some well-known toxicants such as formaldehyde[15], cadmium[1], copper (II)[16], chromium[17] and iron (III)[17]. Furthermore, MFCs have been used to degrade paracetamol in wastewater as a method of water treatment – this technique could indicate the suitability of MFCs for detecting less toxic micropollutants.[18]

Current methods of assessing the bioavailability of a toxicant involve the use of more complex organisms such as fish, algae or daphnia and have long incubation periods (up to several weeks). They are known to have low reproducibility and stability, and require an external transducer to detect the signal from the organism, which is costly and is often a source of error.[1] Determination of toxic compounds is often achieved using lab-based methods, by which a sample is removed from the site and analysed using expensive off-site equipment, such as atomic absorption spectroscopy, ion chromatography or gas chromatography-mass spectroscopy.[11, 19, 20] As well as significant equipment cost, the distance between sampling site and testing location adds undue time delays and costs to the process.[11, 21] Alternatives to these techniques involve site-based test kits which lack accuracy and specificity.[19]

Compared to other biosensors, MFCs have a unique advantage in that they do not require an external transducer, since the presence of a toxicant is immediately recorded as a change in current output of the MFC.[2] Therefore, MFC technology can lead to a cost-effective, simple and repeatable measurement, which can provide rapid screening of biologically active pollutants in water-based environments.[14]

At present, there is a lack of rapid techniques which can analyse the concentrations of these toxicants (and similar) in wastewater. Most often, only total organic content is monitored at regular intervals. Specific analysis of the organic components in wastewater is only performed infrequently as methods are time-consuming and expensive. MFCs show great promise in filling this technology gap, allowing for the detection of specific toxicants, within a timeframe of minutes and at low cost.

Furthermore, there are at present few water treatment technologies which have been demonstrated to effectively remove such compounds from wastewater. It is well documented that they often remain in treated wastewater, at low concentrations (pico- to nano- grams per litre). This is of concern due the lack of knowledge of their chemical

interactions, the effect of them mixing and the hazards this could pose to wildlife or to human life. MFCs could potentially be used to remove these organic micropollutants via their metabolisation. [1, 9]

Therefore, a need to develop tools which can detect and possibly remove this kind of organic pollutant exists in order to protect the environment and public health.

1.1 – AIMS AND OBJECTIVES

The aim of this work is to demonstrate the use of MFCs as biosensors for detection of a range of biologically active organic toxicants, including bisphenol-A, 17β-estradiol, triclosan and diclofenac. These substances have been considered for inclusion onto the first watch list of the European Union's Environmental Quality Standards Directive[6] which assesses the relative abundance and possible environmental impact of emerging micropollutants. All four were on the shortlist, and both 17β-estradiol and diclofenac were included on the final watch list, making them some of the most important micropollutants to monitor. Their chemical structures are shown in Figure 1.

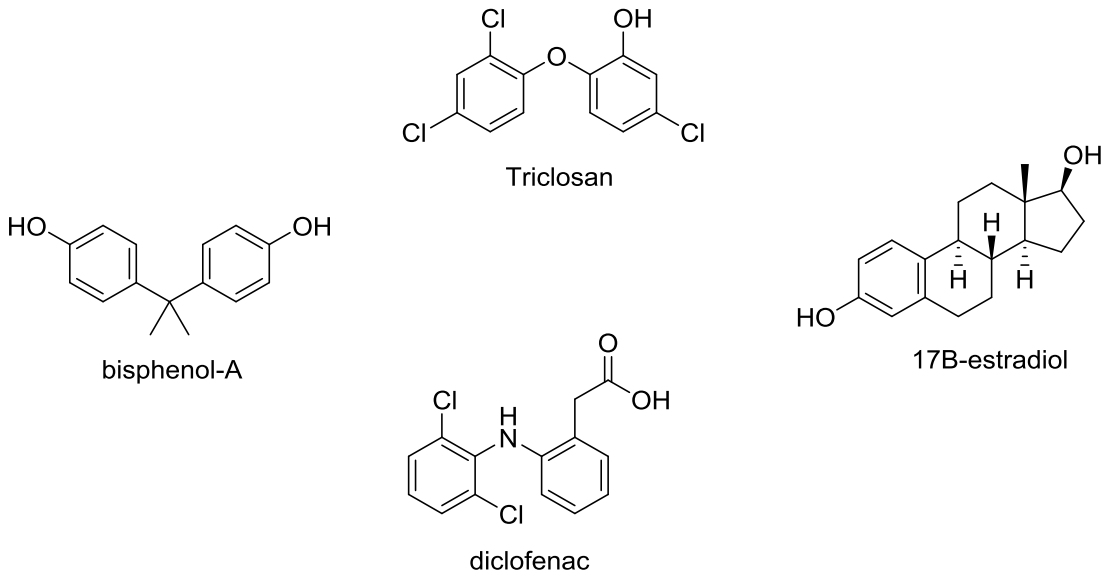

Figure 1 - The chemical structures of bisphenol-A, diclofenac, triclosan and 17β-estradiol

All of these molecules are polycyclic structures, and all of them hydroxyl groups on them. Furthermore, BPA, triclosan, and diclofenac have very similar molecular shapes, meaning they target the same receptors in the human body. [22] In the case of triclosan, its antibacterial properties arise from the multiple chloride groups present, although this effect is not exhibited in diclofenac. – its larger size prevents it from binding to the same receptors.

Diclofenac is a widely-used NSAID – a painkiller and anti-inflammatory drug. It is used to treat a wide range of pain, including that caused by rheumatoid and non-rheumatoid arthritis, traumatic injury and rheumatism.[23] It is most often administered as a sodium or potassium salt, and is known to bind to human serum albumen and is easily metabolised by the human body. Its wide use, presence on the EU watch list for emerging micropollutants,

and previous links to declining vulture populations in Pakistan make it an important target for this study.[6, 7]

17β-estradiol is present in both males and females as a sex hormone, but is more prevalent in women. It is produced in the ovaries and is responsible for acting as a growth hormone for the female sexual organs, and is necessary to maintain oocytes within the ovary. Along with its derivatives, it is often used in oral contraceptive medicines and hormone replacement therapies. It is present in a wide range of species, but has been shown to cause changes in the sexual behaviour, and disruption of the endocrine systems of several fish species when present in water streams.[24-26]

Bisphenol-A is a synthetic, organic compound and is widely used in manufacture of plastics. Although considered safe at levels which presently occur in food (due to plastic packaging) it has been shown to exhibit some hormone-like properties, acting as an endocrine disruptor, similar to 17β-estradiol. As such, it has been linked to the expression of different sex differentiation genes in some species of fish.[27-29]

Triclosan is an antibacterial and antifungal compound which is widely used in consumer products such as toothpaste, soaps, detergents and toys and its use has been common since the 1970s. Despite its frequent use in soaps, it has recently been banned in such products in the United States of America, due to a lack of evidence suggesting it provided any significant benefits over standard soap and water. Its use in consumer products is also restricted in the EU. It has been linked with endocrine disruption, particularly with regard to modulating oestrogen responses in mammals, such as rats, although no evidence exists of this effect in humans. Its usage lifetime is short, as it is often in fast moving consumer goods. As such, it is frequently found in wastewater, and only 97-98% is removed using standard water treatment methods. This can lead to bioaccumulation of the product. [30-32]

As such, the objectives of this work are:

1. To investigate the use of MFCs to detect diclofenac, triclosan, bisphenol-A and 17β-estradiol in an artificial wastewater medium
2. Investigate the potential long-term effects of these 4 micropollutants on the MFCs
3. Determine if these micropollutants can be removed from the inlet feed by metabolisation by the MFCs
4. Examine the use of external control of anode potential can help improve sensitivity towards these 4 compounds, and improve stability of the MFCs.

These four compounds were selected for further study in particular for two main reasons. Firstly, as they were all considered for inclusion on the EU watchlist of emerging micropollutants, this demonstrates the potential environmental impact these compounds could have, and growing international concern about the levels of them in wastewater. This provides a clear rationale for investigating if a specific tool, like MFCs could be used to monitor them, in a simple medium, providing proof of concept.

Secondly, although that watchlist contained many more chemicals, which could have been investigated, it was impossible to study all of them within this work. The four chemicals we chose to investigate further were of similar chemical structures, providing a means of comparison, but were also from different categories of biological molecule – a painkiller, an endocrine disruptor, an anti-bacterial compound and a plasticiser (which also acts as a hormone mimic). This provides a fairly narrow focus, a good frame of comparison between molecules, and also illustrates how chemicals arising from different uses (some in different industries) of similar molecules could possibly be detected.

It is necessary to perform this study in a synthetic wastewater medium, using only a single micropollutant at once. This should be sufficient for proof of concept that MFCs could be used to detect particular concentrations of micropollutants. However, real wastewater is a very complex medium, containing mixtures of many different compounds, and with no control of pH or conductivity, which would make comparing and quantifying results difficult, as they would technically be performed in a medium of different composition. It is also possible that the presence of other compounds in the feed solution may affect the performance of the MFCs in a way that makes it difficult to isolate the effect of the micropollutant alone.

2.0 – BACKGROUND

2.1 – Water Quality and Water Treatment Processes

2.1.0 – Water Resources

Although water covers around 71% of the Earth's surface, it is a scarce resource. Much of the Earth's water, around 96%, is saline, and is in the oceans and seas. Most is not freshwater, and most of that needs treating before it is fit for human consumption. Table 2 shows an estimate of where this water is distributed, and whether it is fresh or saline.

Table 2 - An Estimate of Global Water Distribution[33]

Water source	Water volume (km^3)	Percentage of freshwater	Percentage of total water
Oceans, Seas, & Bays	1,338,000,000	--	96.54
Ice caps, Glaciers, & Permanent Snow	24,064,000	68.7	1.74
Groundwater	23,400,000	--	1.69
Fresh	10,530,000	30.1	0.76
Saline	12,870,000	--	0.93
Soil Moisture	16,500	0.05	0.001
Ground Ice & Permafrost	300,000	0.86	0.022
Lakes	176,400	--	0.013
Fresh	91,000	0.26	0.007
Saline	85,400	--	0.006
Atmosphere	12,900	0.04	0.001
Swamp Water	11,470	0.03	0.0008
Rivers	2,120	0.006	0.0002
Biological Water	1,120	0.003	0.0001
Total	1,409,560,910		

The amount of treated, drinkable water is too small to even be included in these calculations. It is much less, even than the total amount contained in all the Earth's rivers, which is a very low 0.0002%.

The distribution of water over the Earth is not even, and is affected by land features, such as oceans, lakes and rivers, and also by climate. These factors mean that the Earth's population does not have an even supply of water. Therefore, water scarcity is more of a problem in some parts of the world than others – particularly in places with hot climates, often third world countries.[33] About one fifth of the world's population does not have access to safe drinking water, and two fifths are put at risk due to unsatisfactory sanitation. More than one third of the accessible freshwater on Earth is used for agriculture, industry and domestic uses. It is therefore a source of concern that up to 10% of this portion may already be chemically contaminated with biologically active compounds such as pharmaceuticals from domestic use like oestrogen, anti-depressants and anti-inflammatories, industrial chemicals such as hexachlorocyclopen and pentachlorophenol and agricultural run-offs, such as pesticides (*e.g.* Dinoseb, methoxychlor and endothall).[9, 33]

2.1.1 – Water Treatment Processes

As treated water is scarce, it important that we can re-use freshwater. Saline water is potentially treatable by desalination to remove salt and other minerals by several technologies. Aside from treated wastewater, desalination is one of the few rainfall-independent methods of obtaining drinkable water that exist. However, most of these methods are too expensive for operation in normal circumstances, except where other water sources are scarce (*e.g.* in the Middle East and Caribbean).[34] Therefore, this leaves the treatment and re-use of wastewater as the most economical option for obtaining potable water, and water used for irrigation.

For water to be potable, it must be completely free of disease-causing organisms (worms, bacteria, algae, diatoms, fungi, moulds, protozoa, nematodes, and viruses), debris, colour, taste and smell. To that end, water treatment technologies are employed in order to improve the quality of water. Usually, a number of techniques are used in conjunction because none are capable of improving all of these aspects when used alone.

Water treatment technologies are broadly divided into three areas – physical, chemical and energy intensive methods. No one treatment is enough to completely clean water, so they are often used in conjunction with each other. Physical methods rely largely on filtration to remove suspended solids in the water and are one of the most widely used treatment options. There are two different types of filtration technology – one relies on the use of membranes, and is most often used to remove particles of small size, *e.g.* suspended solids. The other relies on beds of sand or activated charcoal to remove pathogens, protozoa and heavy metals. As water is passed through this type of filter, bacteria etc. die off naturally due to a lack of resources to live, or are simply filtered out. Heavy metals and some organic molecules are attracted to the surface of activated charcoal in particular, and can thus be filtered out by adsorption.[35, 36]

Other physical methods include heat treatment, which kills almost all pathogens (with the possible exception of some spores), reverse osmosis, which forces water at high pressure through a membrane impermeable to most contaminants, and distillation, which is the boiling and re-condensing of water to purify it. All of these methods have a fairly large energy cost, particularly due to extended periods of high temperature and pressure. Distillation has a disadvantage in that contaminants with a boiling point less than that of water will not be removed, as they will evaporate with the water. This, however, can be overcome by techniques such as fractional distillation.

Chemical treatments rely on the breakdown of contaminants *via* chemical interaction with a known chemical, as well as the use of chemicals to separate out contaminants for removal by filtration, or the neutralisation of harmful effects of contaminants (*e.g.* altering pH, killing bacteria). They can be used as standalone technologies, but are most often used in conjunction with physical techniques.

The most common chemical decontaminants are chlorine-based. Common examples include sodium hypochlorite and calcium hypochlorite. Generally, the water is "superchlorinated", i.e. far more chlorine is added than strictly necessary. This ensures effective decontamination. The chlorine acts to break down organic material, forming chlorinated compounds, such as trihalomethanes, which can be filtered out using activated charcoal. The leftover, unused chlorine must also be filtered by the same method, or hydrogen peroxide can be added to drive the chlorine off. Chlorination can also kill many microorganisms. The disadvantages of chlorine use include the production of carcinogenic materials upon reaction with organics, which presents a problem with long-term exposure, and a relatively high dose needed to guarantee disinfection – moderate doses are not always effective as this method is highly susceptible to pH and temperature of the water.[35]

Other chemical decontaminants include iodine, which is most often used for small batch treatments. Long-term use is not recommended due to potential problems with causing hyperthyroidism. Some people are also allergic to it, but it is found to be a more effective decontaminant and can be removed easily with sodium thiosulfate. If not removed effectively, it can leave a bad taste. Silver and potassium permanganate have also been recommended for use in the past – both are affective against pathogens (with varying degrees of success) but do not break down organic chemicals. Neither is commonly used in the developing world, due to health problems associated with build-up of silver in the skin, eyes and mucous membranes (argyrosis) associated with high silver intake and high cost/high dosage of permanganate, as well as the pink/brown colour it leaves.[35] Both have been used in the developing world though, were lack of access to alternatives can be a problem.

There are various chemical agents which help to coagulate/flocculate contaminants within the wastewater. The process works by binding organic contaminants, and others, with a metal coordination centre. This forms an insoluble organometallic complex, which can be separated from the water by filtration. The flocculating agent is added to the water and mixed rapidly for 10-15 minutes to allow the formation of flocs of the insoluble material,

which is then left to settle for 10-30 mins (rather than the days needed for normal sedimentation of much smaller particles that have not formed a floc). The most commonly used coagulation agent is alum (hydrated potassium aluminium sulphate). Most of this is removed along with the floc, which also removes some bacteria and viruses with around a 60% rate of removal. This can also help remove colour arising from dissolved contaminants.

The more energy intensive methods of decontamination involve the use of UV-light or ozone. Both are expensive to generate but can directly break down organic chemicals, without leaving any trace of the decontaminant left in the final clean water. They are also successful at killing microorganisms very effectively, in a much quicker time. The lack of residual decontaminant can create a problem if bacteria re-enter already treated water – the water quality will deteriorate more the longer it is stored. Furthermore, both methods compare unfavourably with chlorine in terms of cost.

Ultimately, these methods will be used in whichever combination yields an acceptable result for the intended final use of the water, bearing in mind factors such as cost and availability of reagents. The process chosen will reflect the water's use, in that water for release into rivers does not need to be as pure as that used for irrigation, or for human consumption.[33] A standard wastewater treatment process in the UK would constitute 4 main stages:

1. Pre-treatment of the water – removal of any floating debris using screens, aeration of the water to remove volatile chemicals by the action of bubbling, and filtration to remove the larger particulates
2. Flocculation – to coagulate smaller suspended particles so they can be filtered out with activated charcoal, sand beds or membranes
3. Filtration – A fine filter such as the aforementioned is used to remove the floc, and any remaining colloidal materials in the water
4. Disinfection – Normally through chlorination, to kill microorganisms, prevent re-growth of bacteria and destroy most organic molecules[36]

Despite extensive water treatment systems being in place, treated water is often contaminated with numerous different trace compounds – some of which are natural, but many originate from synthetic sources, such as the agricultural and pharmaceutical industries. These compounds are often persistent in the environment. They are not broken down easily, and hence survive ordinary water treatment processes.[1] These pollutants are known as recalcitrant.

Many of these recalcitrant pollutants are present only in very low concentrations, and are known as micropollutants – in the range of pico- to nanograms per litre. However, many of them pose significant toxicological problems to aquatic life and to human health. In particular, it is their combined effect (co-contamination) that is most concerning, as little is known about their chemical interactions and effect of mixing.[1, 9]

It is the increasingly heavy use of these chemicals for agricultural, industrial and domestic purposes have caused an increased risk of freshwater contamination. It is therefore urgent that methods of sensing these toxic compounds are developed to aid the development of better water treatment processes, and to help understand the effects of these contaminants on aquatic life, and human health.[1]

Water quality must be monitored to ensure the safety of water that is either released into the environment, or treated for drinking/irrigation. At present, water is monitored for a variety of different toxic compounds, ranging from heavy metals, to organic and inorganic pollutants. In different countries, there are different regulations which govern the species to be measured and stipulate the criteria which determine an acceptable level of each contaminant, or group of contaminants. There are a range of techniques employed to detect different species, but many of the techniques are slow, and do not offer real-time measurements.[1, 3, 33, 37] For example the BOD_5 test provides a measure of the levels of organic compounds in wastewater and takes 5 days to complete. Treated water must be stored during this time before it can be released. In the UK, expensive, continuously running analytical equipment is often employed by water treatment companies to determine characteristics such as:

- chlorine residual
- turbidity, colour and clarity
- aluminium content
- iron content
- nitrate levels, and
- pH

These tests rely on an array of probes (such as for pH and chlorine content), spectrophotometry for colour and automated titrations (using expensive reagents in fairly large quantities) to determine these metal contents.[36]

Other contaminants are monitored *via* the collection of samples, which are then analysed in a lab *via* a range of techniques. This manual work is often slow, meaning water has to be stored pending the results of these tests. However, most chemicals are not monitored frequently (i.e. more than once a year, unless they are detected above safe levels.[33, 36]

2.1.2 – Detection of Toxic Metals

Over 40 elements found in the environment are classified as metals. Many, such as sodium, iron and potassium are essential for life, but become toxic in high concentrations. Several, such as zinc, copper, cobalt and manganese take part in important metabolic pathways, or are part of structural proteins within the body. Others have much greater potential for toxicity, for example lead, which is one of the oldest known poisons.[38]

The toxicity of a metal on a living organism is dependent on the dose of the metal, its chemical form (i.e. pure metal, or a metal containing compound), how it is administered (ingested or directly into the bloodstream) and how it is distributed against time (single dose, or several smaller doses over a period of time), and other smaller factors.[39] As such, it is important that a sensor for the detection of such toxicants can detect the concentration of the metal, the species of metal and its concentration against time rather than just the concentration in a sample.

A range of different detection methods for metal ions in water already exist. Most count the total metal content of the sample, but some can differentiate between chemical species, giving a much clearer indication of the toxic effect of the sample. Methods commonly used include atomic absorption spectroscopy (AAS), atomic emission spectroscopy (AES), electrochemical methods, spectrophotometry, and biosensors of several types.

AAS relies on unique absorption of frequencies of light by metals in their ground state. Each metal absorbs light of a unique frequency. The sample must first be atomised and light of a known frequency, corresponding to the metal is shone through the sample. A comparison between the intensity detected through the sample, and through a blank, yields a figure which is directly proportional to the concentration of the metal in the sample.

AES is very similar to AAS, except once atomised, the sample is nebulised, then irradiated with light at a known frequency, causing the excitement of electrons to higher energy levels. As the electrons fall down to lower energy levels, they emit light at a different wavelength, characteristic to each element. Intensity of the emitted light is proportional to the concentration of that element.

Electrochemical methods are concerned with the detection of electrical signals which are indicative of a chemical species or chemical process occurring. Transforms data such as concentration and activity into electrical signals such as potential or current. Examples include cyclic voltammetry, potentiometry and amperometry.

Spectrophotometry uses selective reagents which bind to a metal ion, forming a complex. The complex absorbs light at a characteristic frequency in the UV/Vis region. Light of this frequency is shone through the sample, in a similar way to AES, yielding the concentration of the metal in solution.

Each of these methods has advantages and disadvantages, which are outlined in Table 3**.**

Table 3 - Methods for Measuring Metal Content in Wastewater[2, 37, 39]

Method	Advantages	Disadvantages
AAS	• Can be used on a wide range of metals • Relatively little cost per sample • Low detection limits (*e.g.* down to 1 μg L^{-1} or 8 ng L^{-1}, depending on technique used). • Rapid test • Potential for on-line use	• Not sensitive enough for use on metals contained in biological molecules such as haemoglobin • Very costly equipment • Salts, such as NaCl and phosphates have been shown to interfere with detection • Single element measured at a time
AES	• Most commonly-used spectrometric technique – widely available • High sensitivity • Can measure multiple elements in one test	• Inefficient in terms of energy (for nebuliser) • Easily disrupted by presence of organic species in sample, limiting its application
Electrochemical Methods	• Easy and quick technique to use • Very cheap analysis • Applicable to a wide range of metals • Can detect metals in biological molecules easily, unlike AAS • Highly sensitive	• Complex real-world samples are difficult to analyse selectively by some electrochemical methods. • Electrode surfaces are susceptible to fouling by surface-active materials, damaging the data
Spectrophotometry	• Can be used to measure many trace elements • Can distinguish between different oxidation states of metals, giving more information about the pollutant	• Susceptible to interferences from other coloured substances • Not always an accurate reading as the reagents can form complexes with other things which could be in the sample • Presence of oxidising or reducing agents in the sample can alter the complex formed, causing a completely different reading

2.1.3 – Detection of Non-Metal Contaminants

Non-metal contaminants include microbial contamination and organic/inorganic compounds. In the UK, organic components in wastewater are measured by an array of optical techniques such as UV-Vis spectroscopy, IR-spectrometry and spectrofluorimetry.[40] Each of these techniques is outlined below.

UV-Vis spectroscopy works by measuring the absorbance of light at specific wavelengths in the ultraviolet and visible regions of light. Molecules containing π-bonding or non-bonding electrons absorb light at specific characteristic frequencies, allowing the elucidation of the contaminant in question. The spectrometer measures the intensity of light after passing through a sample and compares it to the intensity before passing through the sample. The change in intensity at a given wavelength is related to the concentration of the compound in question by the Beer-Lambert law. Provided a wide range of light is used, this technique can identify a wide range of organic components at a time, but only those which can absorb in this region.

Infrared spectroscopy is a non-quantitative method of analysis, which allows the detection of certain characteristic bonds in organic structures, such as C=O, C=N, C=C, etc. Each of these bonds are capable of absorbing IR light at a known wavelength and is excited, causing a bond vibration, or stretch. A beam of light of known wavelength is passed through the sample. When the frequency is the same as that of one of these bonds, it is absorbed. Not all bonds absorb this light, as bonds need some degree of symmetry to be able to do so. Since it is a non-quantitative technique, it would only indicate a failure in treatment, the type of molecule that was present (*e.g.* alkene, aldehyde etc.). It is not capable of determining the individual molecule, or its concentration.

The final technique is spectrofluorimetry. This technique relies on the ability of molecules to fluoresce when excited by a high intensity light source at a known wavelength. Each molecule will be excited at a different wavelength. The light is shone at a specific wavelength through the sample. The light given off through fluorescence is measured and is proportional to the concentration of that chemical species in the sample. Not all molecules are capable of fluorescence, however.

A combination of all these tools must be used in order to detect the broadest range of compounds possible. One alternative is to use Gas Chromatography – Mass Spectroscopy (GC-MS), which vaporises the sample before flowing it through a known length column filled with a carrier gas. The solubility of each component in the carrier gas varies – the more

soluble a component, the quicker it will travel through the column. This will allow the separation of the components, which then travel into a mass spectrometer, which ionises them and pushes them through an electromagnetic field in a narrow beam. The amount the magnetic field bends the beam away from from its original course is a function of the ion's mass/charge ratio, allowing calculation of its molecular weight. The retention time in the GC and molecular weight from the MS allow the definite identification of each molecule. This technique, however, is expensive to run and is thus not commonly used for this purpose.

In developing countries, measurements such as biochemical oxygen demand (BOD), chemical oxygen demand (COD) and total organic carbon (TOC) are often used in place of these systems. Whilst they do not detect specific species or groups of molecules, they do provide an alert when treatment has failed. COD provides a measure of the concentration of chemically oxidisable species present in the sample. BOD is similar in function, but more specific since it measures only biologically active organic matter, by comparing the dissolved oxygen content of a sample with that after a 5-day incubation period. TOC measures the total organic carbon content of a sample by acidification, followed by combustion, which releases a measurable quantity of carbon dioxide. These tests are quick and cheap (with the exception of BOD, which takes longer) but are not specific measures.[41, 42]

The most common test for the presence of microorganisms is the total coliform test. Coliform bacteria are those which are rod-shaped, Gram-negative bacteria which do not form spores and can ferment lactic acid. It is the rate of fermentation of a broth of lactic acid (or a substitute) at a known temperature which provides the count of the bacteria.[43]

2.1.4 – Use of Sensors in Water Treatment Processes

A sensor is defined as an electronic device which can detect events or changes within its environment and send that information to other devices (often a computer processor). A sensor is always used in conjunction with other electronics, whether that be a simple light or a complex computer program. There are already some sensors for water quality – some are bio-based, others are not. The main requirements of a sensor for water quality are that it is reliable, repeatable, stable over long term use and cost-effective.[36]

A biosensor for measurement of water quality was proposed as early as 1994 by Vanrolleghem *et al*. A respirographic sensor was constructed to provide on-line measurement of BOD, with one reading being taken every 30 minutes. The response time for toxicity detection was found to be around 1 hour. The sensor detects changes in the rate of respiration of bacteria from anaerobic sludge. Hydrogen peroxide was used as the model toxin in this case and the system is shown to detect its presence over a wide range of concentrations. However, each time a toxic event occurs, the anaerobic sludge in the reactor must be changed. Furthermore, presence of oxido-reduction chemicals can interfere with results. The sensor can, however provide the BOD of a wastewater sample, the toxicity of the test sample towards the sludge and the specific activity of the sludge using simple calculations based on the data it obtains in terms of dissolved oxygen, pH, etc.[44]

Other biological methods, such as bioassays have used a range of organisms such as fish, daphnia and microalgae in order to detect pollutants in waterstreams. Microalgae of several species have been used to detect both organic and inorganic toxicants in a range of different ways. Some sensors are amperometric, and rely on a current being generated to measure the concentration generated. One such example is the work of Naessens *et al.*, in which an algal biofilm was grown upon a Clark electrode. The Clark electrode is specially designed to generate current in relation to the concentration of oxygen it is in contact with. Toxicants such as perchloroethylene were successfully detected, as they inhibited the rate of photosynthesis in the microalgae, which caused less oxygen to be produced.[45]

Other microalgal biosensors used optical or colourimetric measurement to detect toxicants. For example, Lettieri *et al.* have demonstrated that some species of algae generate microstructures, which fluoresce under the presence of certain nitrogen-containing species, such as fertilisers.[46] Other methods of detection with microalgal biosensors include measurement of conductance.[47] Micro-algal biosensors have not yet been commercialised.

More commercial methods of detecting toxicity in water involve the use of daphnia (water fleas) or other organisms such as small fish to determine the toxicity. They use living organisms to detect toxic levels of substances, and note the number of deaths that occur in those animals to see any statistical deviation from the norm. This, however, does not provide any information about what the toxin is, and does not work on non-toxic doses.[14, 36]

2.2 – Emerging Pollutants and Micropollutants

A micropollutant is defined as a pollutant within the concentration range of picograms to nanograms per litre. [1] Although the concentration of each of these toxicants is low, little is known about their effects on aquatic life or human health, especially when their mixing effects and interaction, and metabolisation products are considered.

Many of these micropollutants are also considered "emerging pollutants" (EPs). This term is generally used to refer to "compounds of different origins and chemical nature, which presence in the environment is not considered significant in terms of distribution and/or concentration." [48] However, the frequency with which they are being detected is increasing, and they have the potential to generate an environmental impact and have adverse effects on the health of living beings. [7, 8, 24, 25, 28, 29, 48] Examples of such emerging pollutants include pesticides, pharmaceuticals (antibiotics, analgesics, steroids, antiepileptics, etc.), life-style compounds (*e.g.* caffeine, nicotine), food additives, water treatment by-products (chlorinated organic molecules, metabolisation products), fire retardants, surfactants, hormones and sterols and even ionic liquids.[49]

Freshwater systems worldwide are contaminated by a plethora of these chemicals, originating from both synthetic and natural sources. [1, 9] In European waters, over 700 such compounds have been identified, and have been grouped into 20 classes.[50] Analysis of the influents of two wastewater treatment plants (WWTPs) in Mexico detected 41 and 55 different emerging pollutants present in those two plants alone, once in dry season and once in rainy season.[48] This does not give any information about their presence on a day to day basis – other toxicants may have been present on other occasions. Furthermore, since little is known about their mixing and interaction with each other, or with more common pollutants it is incredibly difficult to begin to predict their possible effect on living beings.

The two WWTPs had different treatment methods in place – the first using only oxidation ditches and UV light lamps, the second used anaerobic/anoxic/aerobic tanks coupled with two disinfection processes: chlorine dioxide and UV lamps. The 2nd WWTP was much better at removing the EPs were present, removing up to 80% of them, while the 1st WWTP could only remove up to 22% of them. This shows that the method of treatment is critical to removing the EPs, but that neither method could remove all the contaminants completely. However, of the 55 toxicants detected, 25 were not removed at all in WWTP 1, and 6 were not removed at all in WWTP 2. This highlights the fact that there may not be a "one-size fits

all" solution for the different toxicants. It is particularly noted that small, polar molecules are not well removed by existing water treatment methods. [49]

In UK wastewater, the most frequently detected EPs are pesticide metabolites (arising from farming), pharmaceuticals (particularly triclosan and carbamazepine), nicotine, food additives and alkyl phosphates.[49] Table 4 shows the results of several studies into the micropollutants found at various UK locations.

Table 4 – Commonly Detected Organic Micropollutants in UK Water

Site	Source of Contamination	Most Common Compounds Detected	Reference
England and Wales	Contaminated and Control Sites	Polychlorinated dibenzo-p-dioxins, Polychlorinated dibenzofurans Present in all samples	[51]
A London river and a rural river	Wastewater Treatment Plant	Ibuprofen, Paracetamol, Salbutamol in all samples. Mefenamic acid (NSAID) in 70% of samples. Propanolol (β-blocker) below limit of quantification.	[52]
Tyne Estuary	Wastewater Treatment Plant	Clotrimazole, Dextropropoxyphene, Erythromycin, Ibuprofen, Propanolol, Tamoxifen, Trimethoprim all quantified. Clofibric acid, Diclofenac, Mefanimic acid, Paracetamol below limit of quantification	[53]
Tees, Mersey, Aire Rivers and Estuaries	Industry	Alkyphenol polyethoxylates detected, above threshold	[54]
Inland Streams	Wastewater Treatment Plant	Ibuprofen, Mefamic Acid, Diclofenac, Propanolol, Dextropropoxyphene, Erythromycin, Trimethoprim, Acetyl-sulfamethazole detected. Paracetamol, Lofepramine not detected.	[55]
Taff and Ely Rivers, Wales	Wastewater Treatment Plant	Trimethoprim, Erythromycin, Amoxicillin, Paracetamol, Tramadol, Codeine, Naproxen, Ibuprofen, Diclofenac, Carbamazepine, Gabapentin and 41 others detected.	[56]
Ouse	Wastewater Treatment Plant	Bisphenol A, Oestrone, 17β-Estradiol consistently detected. Propranolol, Sulfamethoxazole, Carbamazepine, Indomethacin, Diclofenac variably detected. Mebeverine, Thioridazine, Tamoxifen, Meclofenanic acid below limit of quantification.	[57]
UK	-	Diuron	[58]
Stream, Tunbridge Wells	Storm Event, Fruit Growing	Simazine, Diuron, Nonyl-phenols, Endosulfan sulphate, Pendimethalin	[59]
Thames (between 1988 and 1997)	-	Atrazine, Simazine, Lindane	[60]
Eastern England	Wastewater Treatment Plant	Ibuprofen, Erythromycin, Dextropropoxyphene, Diclofenac, Mefanamic acid, Propanolol, Acetyl-sulfamethoxazole, Trimethoprim	[61]

Since such a wide variety of compounds are clearly present in waterstreams, even after wastewater treatment, and so little is known about their effects on aquatic life and/or human life, it is of critical importance that we develop techniques to both measure their presence, and their biotoxicity. [1, 9, 48-50] Until such a robust and sensitive analytical tool is developed, we will be unable to fully understand those impacts on living organisms, or properly assess the risks posed to our freshwater supplies.

2.3 – Detection of Micropollutants in Wastewater – Current Methods

In-situ monitoring of water quality is a particular challenge, and recently, a priority has been placed on detection of organic micropollutants such as pharmaceuticals in recent years. [62] In most cases, water samples are collected from the field at various and are brought back to the laboratory where they are analysed. Useful information is obtained by this periodic analysis, but it is widely recognized that this method is inadequate in terms of both spatial and temporal resolution. To alleviate the problem of undersampling, research has shifted towards the development of sensors that allow continuous in situ monitoring over long periods of time.

Current methods in industry rely on this periodic sampling and then analysis by techniques such as LC-MS. LC-MS first involves separating out the constituent compounds in a sample using liquid chromatography. Each compound has a different solubility in the liquid phase and as such, travel along a set distance of a silica-packed column at different speeds before they are eluted out of the column. The compounds are separated based on this elution time, before each fraction is fed into a mass spectrometer. The mass spectrometer first ionises the molecules in each fraction, giving them a charge. The ions are fired in a single beam towards a detector, where they encounter a magnetic field. The magnetic field alters their path of flight, depending on their molecular mass and their electrical charge. They hit the detector at different locations, allowing their mass and charge to be elucidated. The information on elution time and the mass/charge ratio of each fraction allows its molecular identity to be determined. The process is expensive and requires specialised equipment. Furthermore, it cannot provide online, or *in-situ* measurement of the levels of organic toxicant present. The result is generally obtained a few days after the sample has been taken. [39]

It has been shown that chemical sensors and portable analysers play a pivotal role in the monitoring of oceans, lakes, and rivers. In particular, sensors for dissolved oxygen, conductivity, and pH are now routinely used to monitor and understand the aquatic environment. If such a technology could be developed that would detect the levels of organic compounds in a sample, this could revolutionise the way water quality could be monitored. [63]

Although no such technologies have yet been widely used in industry, it has been demonstrated that continuous monitoring could be provided by infrared optical sensors,

which can detect a range of organic micropollutants, with a high degree of molecular specificity. Broadly, there are two types of optical infrared sensors: (i) those which seek to directly detect the presence of a compound based on its molecular properties such as absorption of light of a given wavelength and (ii) those which seek to elucidate the presence of a molecule indirectly via the emission or absorption of light at a given wavelength during a chemical reaction. These are known as opto-chemical infrared sensors.

It has been demonstrated that such technologies are suitable for *in-situ* monitoring, and offer a high degree of molecular specificity, even when mixtures of organic compounds are present, as each organic molecule possesses a unique UV spectrum. However, their sensitivity needs improvement in order to detect down into the low μg L^{-1} concentration range. [62, 63]

Biosensors have been shown to be another alternative technology for the detection of toxic compounds in water. Biosensors can be relatively inexpensive methods of monitoring water, and their compact design could make them portable and suitable for onsite and online use. They could also potentially be disposable. [64] However, the use of an external power source and costly equipment increases the cost to some extent, and limits their use in remote or long-term environmental monitoring.

Most biosensors are enzymatic and operate *via* electrochemical means. The IUPAC definition of an electrochemical biosensor is a "self-contained integrated device, which is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element which is retained in direct special contact with an electrochemical transduction element".[65] In the case of microbial fuel cells, the recognition element is the enzyme used, which is attached to the electrode, which acts as the transducer.

They have a very high selectivity towards the target analyte, arising from the recognition element's unique design. However, they have major drawbacks, as the use of enzymes is time consuming and costly, and immobilisation protocols of these enzymes are long. Furthermore, the resultant biosensors have a short life time and poor stability, due to the deactivation of enzymes. [64]

Some work has focussed on the use of whole bacteria rather than individual enzymes. These are commonly known as whole-cell biosensors. The use of bacteria removes the difficult and costly processes associated with the use of enzymes, instead requiring only a simple process

to grow a biofilm. [66-68] Microbial biosensors could be more versatile and sensitive to a large variety of analytes, thanks to the large range of enzymes that within their cells. That said, particularly where mixed bacterial cultures are present, their sensitivity to a wide range of toxicants could become a problem when complex media are analysed.[66] Electrochemical approaches such as amperometry or potentiometry must usually be used with microbial sensors in order to detect the signal *via* a change in current or potential difference, but it is also possible to use optical methods to detect a colour change *via* an electrochemical reaction, or an absorption of a wavelength of light.[62, 69, 70]

Examples of such devices include work by Ben-Yoav *et al.* described a biosensor based on a bacterial biofilm that was shown to detect DNA-damaging compounds in water. They used a genetically engineered *E. Coli* strain to grow a biofilm which they integrated on a microchip and used a whole-cell biosensor to detect nalidixic acid. The biofilms were exposed to different concentrations of nalidixic acid and a substrate and underwent chronoamperometry. The electrochemical signal generated was found to be proportional to the concentration of the acid present. [67] The chips had a long shelf life compared to similar tests, and were found to be suitable for use in harsh field conditions. However, they were only suited to detecting toxicants which inhibited the action of a specific enzyme in the cell, although this could be overcome by developing different strains of bacteria for different applications.

Work by Si *et al.* developed whole cell electrochemical biosensors to detect fumarate. Fumarate is an organic molecule associated with the metabolisation of various compounds as well as food spoilage. The device they developed reduced fumarate at the electrode surface, and therefore provided an inward electron flow to a biofilm of *S. Oneidensis*. That is to say electrons were passed from the electrode to the bacteria during the reduction fumarate. The more fumarate was present, the more electrons were passed to the biofilm. The biofilm was then monitored using chronoamperometry, poised at -0.6 V, and a stable current output was reached. During dosing of fumarate, the current output of the device would increase, and this increase was proportional to the dose of fumarate. The device was tested against other sugars and simple carbon-based molecules and it was found that it did not respond in the same way to these sugars – the response was much smaller.[68]

Devices like these provide rapid and cheap measurements of the concentration of a given toxicant in wastewater, but they require a potentiostat to control the anode potential. Furthermore, they are only suited to detecting very specific toxicants, although in the case

of a microchip, different test chambers could be used to detect different toxicants in a single use. The use of microbes that can survive harsh conditions such as pH, salinity and temperature extremes make an attractive proposition compared with enzymatic tests or bioassays. However, the full deployment of microbial biosensors is faced with various challenges, as such sensors often have low selectivity and/or low detection limits.[11, 67, 68] In the case of single cultured biosensors, risk of contamination with other microorganisms could pose a further significant threat.[11]

One type of biosensor is based on Microbial Fuel Cells, which show great promise as biosensors for biologically active compounds in water.

2.4 – Microbial Fuel Cells

Microbial fuel cells (MFCs) are a variation of fuel cell in which electrogenic microorganisms are employed to generate electrons (e^-) and protons (H^+) during the metabolism of organic substrates. These electrons are passed across the cell membrane to the anode and run through an external circuit, generating a current. This process is known as extracellular electron transfer.[71] The protons are transferred through the proton exchange membrane (PEM) and combine with the oxidant (usually oxygen from air) to form a reduction product (see Figure 2).[11] In some cases, another chemical oxidant, such as ferricyanide or Mn (IV) are used but these must either be replaced or regenerated. In the presence of air, the electrons generated would be fed to molecular oxygen, but under anaerobic conditions, they reach the anode surface, generating electricity.[72] Bacteria are usually be used at the anode, but algae have also been used in some cases as the organism. [47, 72, 73]

The microbially catalysed electron generation at the anode and subsequent consumption of these electrons at the cathode are the defining characteristics of an MFC, provided both are sustainable. Systems using, for example enzymes, catalysts or sacrificial anodes would not qualify as MFCs, as they do not require the presence of a microorganism on the anode to carry out the metabolisation of the fuel source.[72]

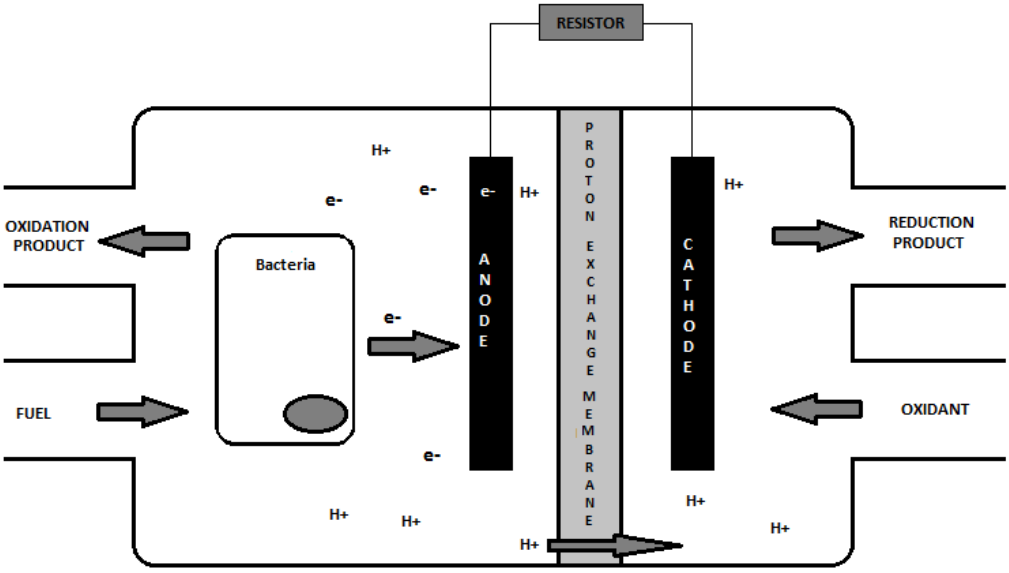

Figure 2 - Schematic Diagram of a Microbial Fuel Cell (adapted)[73]

Several different configurations are possible when designing MFCs exist. The most widely used is a standard H-shaped reactor, as shown in the centre of Figure 2. This consists of two chambers, connected by a tube, but separated by a Proton Exchange membrane such as Nafion. It is important when using this design is to choose a membrane which allows the passage of protons between the chambers, but ideally also blocks the passage of the substrate or electron acceptor to the cathode chamber. A salt-bridge can be used instead of the membrane, but MFCs using a salt bridge produce little power due to high internal resistance. [72] The anode and cathode are placed in each chamber, and an electron acceptor solution added to the cathode side, and the substrate solution to the anode side. These fuel cells are generally only suitable for laboratory based testing of materials etc, as they operate in a limited batch mode, and require constant replenishment of the electron acceptor.

It is not necessary to submerge the cathode or place it in a separate chamber, as long as it is still in contact with some liquid and air can be used as the electron acceptor. These designs consist of a single chamber. The cathode must be exposed to air on one side, and the liquid feed on the other. The cathode is sealed into the cell on the opposite side of the chamber. The addition of a membrane at the cathode to these systems helps to reduce oxygen travelling to the anode. [72] The advantage of this kind of design is that it lends itself easily to continuous flow operations, and does not need constant replenishment of the electron acceptor. This makes it more applicable to settings outside the lab, as a continuous feed of electron acceptor is not required.

The substrate used can be any organic matter, such as glucose, sucrose or acetate, or biodegradable organic-rich wastewaters, such as municipal waste, industrial or agricultural wastewaters. These waste resources would be ideal for use as a fuel source, as they are very sustainable, and available in large quantities. They take a fuel that is essentially free and unwanted, and turn it into valuable electricity. In the case of wastewater, while providing a source of energy, it would also be cleaned up during the process, perhaps creating an economically viable method of coupling up the two processes, whilst also greatly increasing the sustainability criteria of such a device. Compared to wind/solar energy, MFCs offer a more reliable base load, and compared to fossil fuels, they use sustainable, eco-friendly fuels. They also run at low temperatures, have very low pollution levels (as the only waste product is water) and run on a waste fuel source. Compared to fossil fuels they have the following advantages:

- Sustainable, eco-friendly fuel sources
- No fuel storage issues
- Inexpensive catalysts
- Reliable base load power
- Flexible reactor design
- Mild operation conditions
- Potential to treat wastewater
- Low pollution level
- Cost-efficiency is favourable
- Can be scaled up to integrate further technologies.[74]

Bacteria in the MFC catalyse the degradation of organic material in the water, releasing electrons which are then transferred to the anode, generating a current. [2] The current output correlates to the concentration of organic substrate in the water, allowing the amount of organic matter to be calculated.

As such, MFCs have been developed for used as biosensors to test for toxicity from a range of compounds based on a comparison of the current output before and after dosing of a toxin such as a toxic metal or organophosphorous compounds,[2, 4, 14, 75, 76] and for the determination of biological or chemical oxygen demand (BOD or COD), which is an indicator of the efficacy of a water treatment process.[3, 77, 78]

Compared to enzymatic fuel cells, which use redox enzymes alone (rather than whole organisms), MFCs are found to be more robust for long-term applications, because they are

self-growing and renewing, meaning they have a greater longevity, whilst enzymes alone can be fragile and easily poisoned, damaged or blocked.[79] Sensors based on microbial fuel cells have been shown to be stable for at least 5 years, with minimal maintenance, which is comparable to other electrochemical sensors.[3]

A unique advantage of microbial fuel cells is that they do not require the use of an external transducer, which lowers their cost compared to other biosensors.[1] This is because the presence of a pollutant in the feeding stream is immediately recorded as a change in the electrical output of the device.[1, 2]

Despite the above advantages, and the promise of multiple applications, their performances have often been too low for scale-up power generation devices to be economically viable.[79, 80] However, the use of MFCs as the basis for biosensors by generating an electrical current upon the metabolism of a certain substrate (*e.g.* glucose) is more promising, for example, to determine the biological oxygen demand of wastewaters, which is a requirement for the safe disposal of sewage.[37, 74, 76]

2.4.1 – MFC Theory

MFCs operate *via* the metabolism of a carbon-based fuel source, such as acetate or glucose. The half equations for the breakdown of acetate are shown below.

$$\text{Anodic Reaction: } CH_3COO^- + H_2O \xrightarrow{\text{microbes}} 2CO_2 + 2H^+ + 8e^-$$

$$\text{Cathodic Reaction: } O_2 + 4e^- + 4H^+ \longrightarrow 2H_2O$$

In the anodic reaction, electrons are generated, which are those used to generate an electrical current, by flowing them through an external resistor, which generates the current according to Ohm's Law ($E_{cell} = IR_{int}$). This means that in effect, the external resistance is the controlling factor in terms of the magnitude of the current generated (assuming all other factors are constant). When they are not constant, the current can be affected as a result.

The rate at which electrons are generated is controlled by the rate of bacterial metabolism, and this speed of this metabolism is affected by factors such as temperature, pH and concentration of the fuel source, and can be increased or decreased by the presence of certain toxicants.[1, 2] However, with careful control of pH, temperature, etc. it has been shown that MFCs have a linear response in current output *vs.* concentration of fuel source.[81] This linearity is disrupted by the presence of toxicants, and it is this change in metabolism

which causes a detectable change in current output from the MFCs, making them suitable for use as biosensors for toxicants.

As well as affecting the metabolism of the bacteria, temperature can affect the current output *via* Nernst equation, shown in Equation 1.

$$E_{cell} = E^{\ominus}_{cell} - \frac{RT}{zF} \ln Q$$

Equation 1 - The Nernst Equation

E_{cell} is the cell potential at the temperature of interest, $E^{\ominus}_{cell}$ is the cell potential under standard conditions, R the universal gas constant, T the temperature of interest, z the number of moles of electrons produced in the cell reaction, F is Faraday's Constant, and Q the reaction quotient. As a result of this relationship, when temperature changes, the cell potential is affected. This leads to a change in current output of the MFC, as a result of Ohm's Law, once again.

The Reaction Quotient for a general chemical reaction in which α moles of a reactant A and β moles of a reactant B react to give σ moles of a product S and τ moles of a product T ($\alpha A + \beta B \rightleftharpoons \sigma S + \tau T$) is given by Equation 2.[82]

$$Q_r = \frac{\{S_t\}^{\sigma}\{T_t\}^{\tau}}{\{A_t\}^{\alpha}\{B_t\}^{\beta}}$$

Equation 2 – Reaction Quotient

These methods rely on external resistance being controlled to create a steady current output, against which to compare any signal drop. However, control of external resistance is not the only method of controlling the cell potentials. Work by Stein *et al.* has reported that of MFCs acting as biosensors for toxins can be controlled by not only external resistance, but also by anode potential and electrical current, with different effects on the sensitivity. In all cases, cell potential was measured using a voltmeter and converted into currents.

When external resistance was controlled, it was found that low external resistances yielded low baseline currents, but the biggest change in signal from the MFC when a toxic event occurred, however a large recovery time for the cell was needed. A high external high resistance caused a decrease in the sensitivity of the MFC, but higher baseline currents and a much shorter recovery time was needed.

To control anode potential, a potentiostat was used. High sensitivity only occurred at high anode potentials (>-0.4 V). Similarly, when a galvanostat was used to control the electrical current, high sensitivity was only seen above 0.5 mA. A long recovery time was needed in both cases for the system to return to steady state. The study concluded that external resistance was the optimal method of controlling the system, as it allowed bacteria to adjust to both the anode potential and current, meaning a quicker recovery time was observed than when they could only adjust to one of these factors (as per the other control methods).[75]

Another factor which can affect the performance of MFC-based biosensors is internal resistance, which can be reduced by miniaturisation of MFCs (see Section 2.4.2). However, small sized MFCs have been shown to have large internal resistance in some cases, particularly in the presence of membranes.

Stein *et al.* produced models which describe the effect of toxicants on the kinetics of microbial fuel cells. They describe the rates of reaction at the bio-anode as essentially comprised of two separate rates – biochemical rates which account for the metabolism of the substrate into to a new product, and electrochemical rates, which determine the rate of transport of the electron to the anode, and into the circuit.

The starting point for their kinetic model is the standard Butler-Volmer-Monod equation, as seen in Equation 3.

$$I = I_{max}\frac{1 - e^{-\eta f}}{K1.e^{-(1-\alpha)\eta f} + K2.e^{-\eta f} + \left(\frac{Km}{S}\right) + 1}$$

Equation 3 – The Butler-Volmer-Monod Equation (BVM)

where I_{max} (mA) is the maximum current determined by maximum enzymatic rates of microorganisms, η is the overpotential (V), *K1* is a lumped parameter describing the ratio between biochemical and electrochemical rate constants, *K2* is a lumped parameter describing the forward over backward biochemical rate constants, *Km* (mol/l) is substrate affinity constant, *S* (mol/l) is the substrate concentration, f is equal to *F/RT*, where *F* is Faraday's constant, *R* the gas constant and *T* is the temperature. This model contains no toxicity term, so does not take into account the effects of any toxicant on the electrochemical processes in the cell.

Four models were proposed to help try to model the effects of toxicants. All were based on the BVM equation, each containing χ_i, the concentration of a toxic component (mmol /l) and

K_i, the affinity constant of that toxicant to the microorganism. A smaller K_i indicates a higher toxicity to the organism.

The first model assumes the toxicant is a non-competitive inhibitor or irreversible inhibitor. The model is named "Itox" and is shown in Equation 4.

$$I = I_{max}\frac{1 - e^{-\eta f}}{K1.e^{-(1-\alpha)\eta f} + K2.e^{-\eta f} + \left(\frac{Km}{S}\right) + 1} \times \frac{K_i}{K_i + \chi_i}$$

Equation 4 – Itox Model

The second model is based upon a scenario whereby the substrate is not fully oxidised at the anode, and is instead partially oxidised by the toxicant. This leads to a change in *K1*, which is the ratio between the biological and electrochemical rates. In this case, the model is known as "IK1" and is shown in Equation 5.

$$I = I_{max}\frac{1 - e^{-\eta f}}{K1.\left(\frac{K_i + \chi_i}{K_i}\right).e^{-(1-\alpha)\eta f} + K2.e^{-\eta f} + \left(\frac{Km}{S}\right) + 1}$$

Equation 5 – The IK1 Model

The third model refers to a situation when the toxicant causes no more product to be formed, much more product is formed, or when the toxicant is an acid. These situations cause *K2*, the ratio between the forward and backward reaction rate constants of substrate oxidation, to change. This model is known as the "IK2" model and is shown in Equation 6.

$$I = I_{max}\frac{1 - e^{-\eta f}}{K1.e^{-(1-\alpha)\eta f} + K2.\left(\frac{K_i + \chi_i}{K_i}\right).e^{-\eta f} + \left(\frac{Km}{S}\right) + 1}$$

Equation 6 – The IK2 Model

The final model proposed allows for a situation where there is competition to bind with the redox enzyme in the microorganism between the substrate and the toxicant, resulting in an altered substrate affinity constant (*Km*). This model is called the "IKm" model and is shown in Equation 7.

$$I = I_{max}\frac{1 - e^{-\eta f}}{K1.\,e^{-(1-\alpha)\eta f} + K2.\,e^{-\eta f} + \left(\frac{Km}{S}\right)\left(\frac{K_i + \chi_i}{K_i}\right) + 1}$$

Equation 7 – The Ikm Model

It was found that all of these models produced accurate simulations of the polarisation curves of MFCs, provided that the toxicant used was of the right type for the model. In terms of their application in the real world, however, they are limited by a lack of information on K_i & χ_i values. The authors suggest a database could be set up to allow these models to be widely used.[83]

2.4.2 – Electrochemical Characterisation of MFCs

MFCs have been characterised *via* several different electrochemical methods as indicators of their performance. The most common is a polarisation curve, in which a plot of the current output against potential difference is obtained *via* use of a potentiostat.

They allow the calculation of the power output of the fuel cells against the current, as well as the calculation of the optimum external resistance to be used to generate maximum power, through Ohm's Law and other simple power equations, such as $V = W / I$. [13]

A potentiostat is used to control the potential difference between two electrodes whilst measuring any flow of current between them. Though the potential difference and current observed can be measured between the same two electrodes (a working electrode (WE) and counter electrode (CE)), if analysis of a single electrode is required it is necessary to use a third electrode (a reference electrode (RE)) into the system. The reference electrode is isolated from the bulk solution by a salt bridge. This is necessary in order to remove the effect of the potential measured between the two electrodes of interactions between the CE/electrolyte and the electrolyte itself.[84] This three-electrode cell setup is the most common electrochemical cell setup used in electrochemistry, due to its reliability, however a two-electrode setup can also be used in some circumstances. A schematic diagram of this setup is shown in Figure 3.

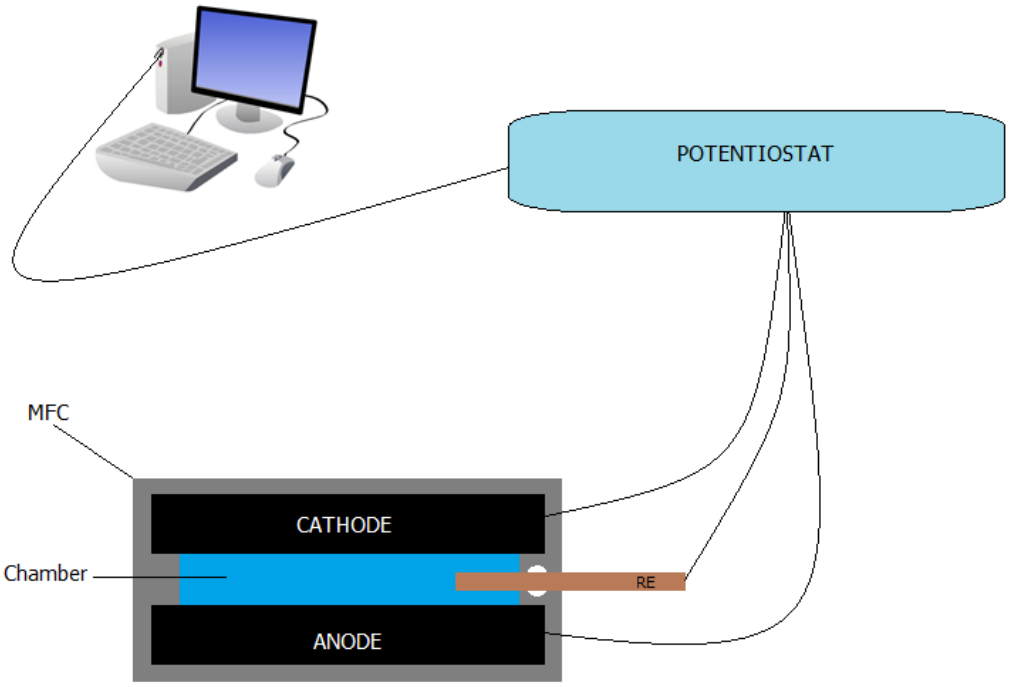

Figure 3 - Experimental Setup for Electrochemical Characterisation Experiments. The chamber contains fresh feed solution.

The current flows between the CE and the WE. The potential difference is controlled between the WE and the CE, whilst it is measured between the RE and WE. The potential between the WE and CE is not measured during most techniques, as it is the potential applied by the system and it is adjusted in order that the potential difference between the WE and RE will be that which is specified. The techniques used in this study are outlined in the following sections.

2.4.2.1 – Cyclic Voltammetry

Cyclic voltammetry (CV) is a technique which is useful in determining the capacitance of an electrode in solution. CV scans are performed using a potentiostat in a 3-electrode configuration. The potential applied between the electrode of interest (in this case the anode) and the CE (in this case the cathode) is varied such that the potential difference between the WE and the RE varies at a constant rate (the scan rate, v) which is set by the user. The resulting current signals (measured between the WE and CE) corresponding to this change in potential difference are then recorded to create a CV curve, also known as a voltammogram (see Figure 4).

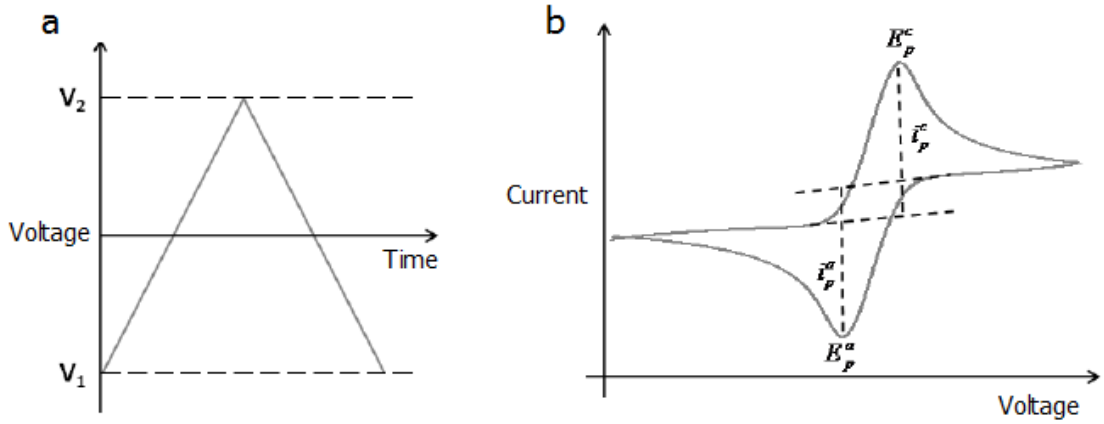

Figure 4 - A CV Waveform (a) and a typical CV response (b) [85]

During a CV scan the electrode-solution interface behaves in the same way as a parallel-plate capacitor. Charge accumulates on one side of the capacitor when a potential is applied across it, as in Figure 5. [86]

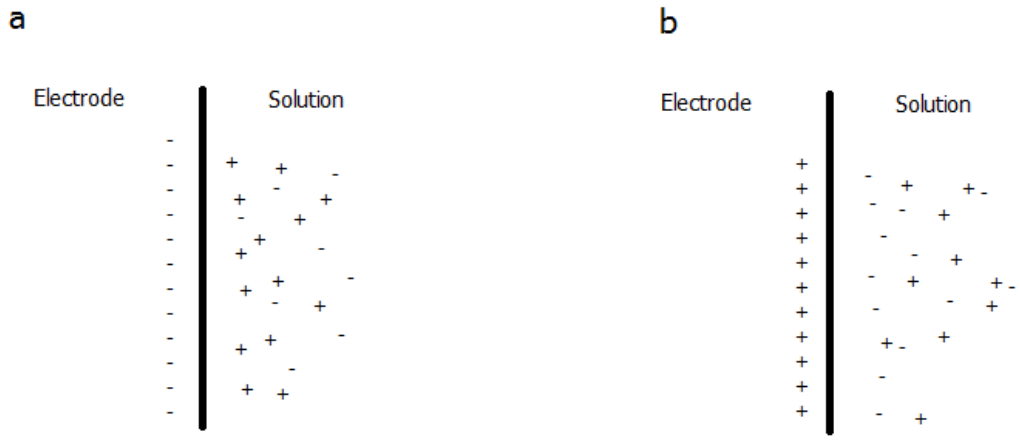

Figure 5 - Electrode-electrolyte interface acting as a capacitor with a charge on the metal, Q, (a) positive and (b) negative

The accumulated charge leads to the flow of a charging current as electrons flow between the electron rich plate, and the electron poor plate. The charge on a capacitor consists of the sum of the excess of electrons on one plate and the deficiency of electrons on the other. Similarly the capacitance, *C*, of an electrode in solution is due to the build-up of charge due to an excess or deficiency of electrons on the electrode in question.

For a simple parallel plate capacitor, charge on the capacitor, *Q*, is proportional to the voltage drop across the capacitor, *E*:

$$Q = CE \qquad \text{Equation (8)}$$

Assuming that capacitance is constant, the magnitude of the charging current can be calculated by differentiating this equation with respect to time, *t* to give:

$$\frac{dQ}{dt} = C\frac{dE}{dt} \qquad \text{Equation (9)}$$

Since $\frac{dQ}{dt}$ is an expression for current, *I*, and $\frac{dE}{dt}$ is the scan rate, *v*, the charging current is simply obtained from:

$$I = Cv \qquad \text{Equation (10)}$$

The charging current can be obtained by measuring the difference in anodic and cathodic current observed, *ΔI*, during a CV scan, as shown in Figure 6.

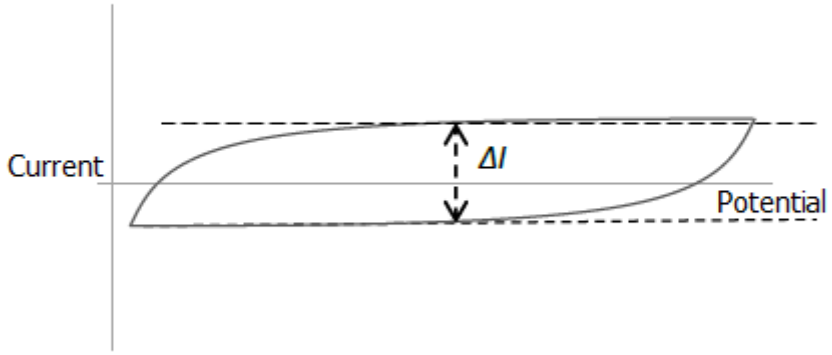

Figure 6 - An illustration of a CV scan showing the charging current, ΔI

2.4.2.2 – Polarisation Curves

Polarisation curves are a plot of cell potential against current density, which are used to assess the performance of a fuel cell. Theoretically, the potential difference of a fuel cell should be the difference between the potential at which the fuel can be oxidised and the potential at which the oxidant can be reduced – the open circuit potential (OCP). However, in reality, the operating potential difference of a fuel cell is always less than this value due to an effect known as polarisation. [87]

Polarisation is the reduction in potential difference when a current is flowing, and is caused by the presence of an overpotential (overpotential is the potential difference between a

half-reaction's thermodynamically determined reduction potential and the potential at which the redox event is experimentally observed). There are three possible sources of overpotential: the activation overpotential, the ohmic overpotential, and the concentration overpotential. Activation overpotential is caused by the flow of current which occurs due to a shift away from the equilibrium position of the anodic and cathodic half reactions. The ohmic overpotential is the result of the sum of the ionic, electronic and contact resistances and is governed by Ohm's law. The concentration overpotential is the result of mass transport limitations in the system, which arise when higher current flows result in the depletion of reactants at either the anode or cathode.

Polarisation curves are often coupled with power density curves, which show at which potential and cell resistance the power is maximised. The power curve can be generated from the same data as the polarisation curve using basic equations. This gives very useful information on the parameters required in order to maximise a fuel cell's performance.

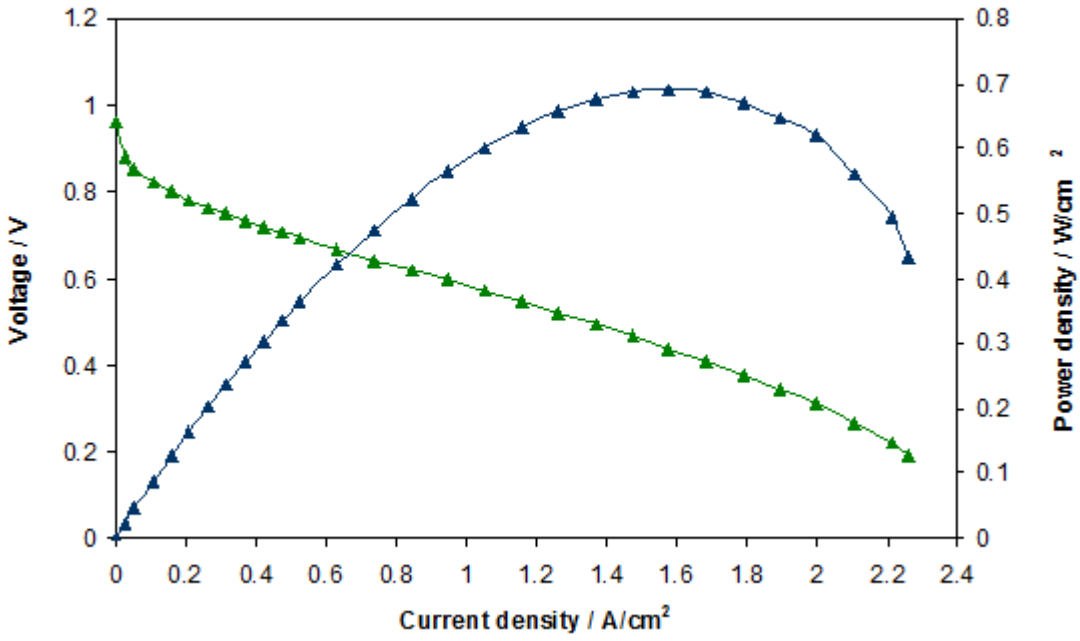

Figure 7 - Typical Polarisation and Power Curves[88]

Polarisation curves cannot give any information about individual components of a system, as CV could, but are passive methods of monitoring current flow, since they do not require a current to be applied. This makes them much less likely to damage the biofilm or bacteria

within during analysis. They instead give indications on the sum total inefficiencies of the system.

2.4.2.3 – Electrical Impedance Spectroscopy (EIS)

Another method of characterising MFCs is that of electrical impedance spectroscopy (EIS). This technique provides a measure of the opposition that a circuit presents to a current when a voltage is applied. This current causes the voltage and current to be out of phase with each other as they increase and decrease, giving a "phase angle" between them. It is a similar concept to resistance, relating to the ratio between the voltage and current in an alternating current circuit and is measured in Ohms. Resistance can be thought of as electrical impedance with a zero phase angle, and in an ideal resistor impedance is equal to resistance.

It is measured by applying a sinusoidal voltage to the device, in series with a resistor, and measuring the voltage across the resistor and across the device. The frequency of the voltage is swept between a range of values during measurement, providing the phase angle and the magnitude of the impedance.[89] A simulated example of the output is shown in Figure 3.

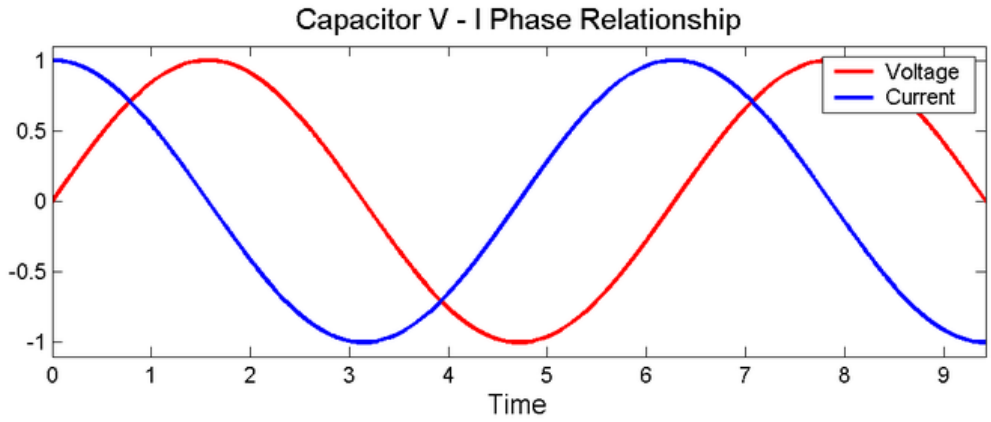

Figure 8 - Simulated EIS Results for a capacitor, created in MATLAB by Jeffrey Philippson

Electrochemical Impedance Spectroscopy is used in a wide range of applications. It is used in the paint industry to measure the thickness of coatings, and has a wide variety of applications in the food industry. It was initially applied to the determination of double-layer capacitance, but has since proven a useful technique in the field of biosensors, for determining biofilm thickness. In fuel cells, it is a commonly-used technique to determine the internal resistance of the system, amongst other electrochemical characteristics.

EIS measures the impedance of a system over a range of frequencies, which reveals the frequency response of the system, including the energy storage and dissipation properties.

Impedance is the opposition to the flow of an alternating current (AC) in a complex system. Usually, a complex electrical system comprises both energy dissipating (*e.g.* a resistor) and energy storing (*e.g.* a capacitor) elements. If the system is purely resistive, then the opposition to AC or direct current (DC) is simply resistance. [90]

This technique has grown tremendously in stature over the past few years and is now being widely employed in a wide variety of scientific fields such as fuel cell testing, biomolecular interaction, and microstructural characterization. Often, EIS reveals information about the reaction mechanism of an electrochemical process: different reaction steps will dominate at certain frequencies, and the frequency response shown by EIS can help identify the rate limiting step. [90]

In MFCs, it is particularly useful in order to determine the internal resistance of the system, which is one of the factors which can determine the power output of the devices. Furthermore, studies have investigated how thickness of the biofilm can be determined by EIS. It can also be used to check the health and activity of a biofilm by detailing its electrochemical characteristics. [90]

To perform these experiments, the MFCs were connected to the potentiostat, and were filled with fresh feed solution before analysis, as in Figure 3. The electrodes were connected to the impedance analyser rather than the usual connections on the potentiostat, however.

An alternating voltage 0.01 V of was applied at a frequency range between 100000 and 0.1 Hz. The data gathered was interpreted *via* the NOVA 2.0 software provided with the potentiostat. Bode plots of Frequency *vs.* Phase (to determine the phase shift) and Frequency *vs.* Magnitude (to determine the magnitude of the frequency response) were created. (See Figure 9)

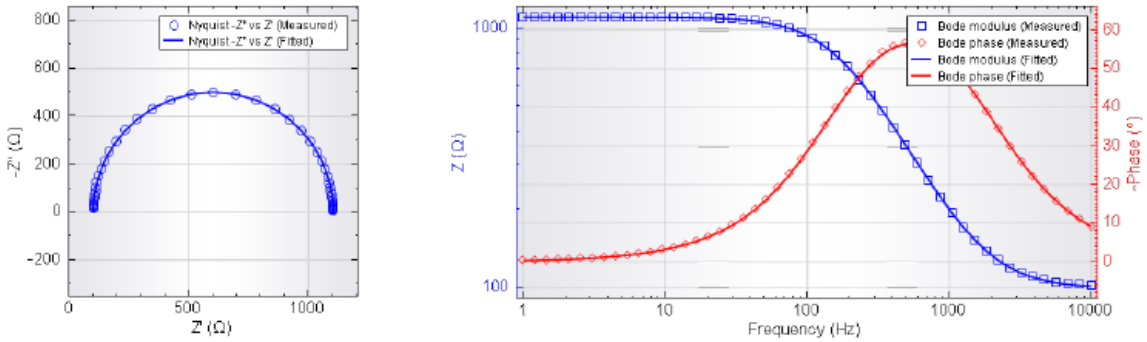

Figure 9 - Typical Nyquist (left) and Bode (right) Plots (generated using Tutorials in the NOVA software)

A Nyquist diagram was also created, showing real impedance (Z) against imaginary impedance (Z”). The data in this diagram was processed using a semi-circle fitting tool in the NOVA 2.0 software to generate the equation for any circle-like area of the graph. After the mathematical fitting, the circuit parameters were inputted to the software, and used in order to calculate the value of internal resistance.

2.4.3 – Miniaturisation of MFCs

Most research to date has been carried out using devices with chamber volumes ranging from several millilitres to litres. [71] Aside from being easier to work with on a laboratory scale, those with volumes on the millilitre scale (mini-MFCs) are easy to fabricate and versatile in terms of application.

The miniaturisation of MFCs allows for improvements in efficiency of the cell by reducing internal resistance and improving mass transport. Internal resistance (R_{int}) is defined as the resistance within the fuel cell given by $E = E_b - R_{int}I$ where E is an external potential difference (electromotive force), E_b the observed potential difference between anode and cathode and I is the current.[91] This is most easily obtained by recording of a polarisation curve of the fuel cell. The reduction in internal resistance experienced by mini-MFCs arises from the increase of the surface area to volume ratio of the electrode compared to the volume of the chamber.[91] Mass transport is improved in these mini-MFCs as a result of the shorter distance between anode and cathode, reducing the distance necessary for migration and diffusion of particles involved, improving the kinetics of the reaction according to Fick's second law of diffusion.[72, 92]

To date, the best ever performances of microbial fuel cells have been achieved using small scale devices. [71] Recently, much improved power densities have been achieved using mini- and micro-MFCs. However, size of the chamber is not the only factor involved in determining the power output. Electrode material and other factors, such as the substrate and catholyte can also play a role, as **Table 5** demonstrates.

Table 5 – A Summary of the Performance of Some mini- and micro-MFC (adapted)[71]

Anode Chamber Volume (µL)	Innoculum	Anode Material	Substrate	Catholyte	Open Circuit Voltage (V)	P_{max} (W m^{-3})	I_{max} (A m^{-3})
2500	Mixed Bacterial Culture	Carbon Cloth	Acetate	Air	N/A	1010	5050
1200	*Shewanella oneidensis* DSP-10	Graphite Felt	Lactate	Ferricyanide	0.7	660	1800
650	*Shewanella oneidensis* MR-1	Gold	TSB	Ferricyanide	0.51	0.025	0.34
650	*Shewanella* sp. Hac353	Gold	TSB	Air	N/A	0.17	5.5
400	*Shewanella oneidensis* MR-1	Graphite Felt	Lactate	Ferricyanide	0.65	4400	N/A
16	*Saccharomyces cerevisiae*	Gold	Glucose	Ferricyanide	0.3~0.5	0.5	1000
15	*Saccharomyces cerevisiae*	Gold	Glucose	Ferricyanide	0.49	32	302
10	*Shewanella putrefaciens*	Gold	Lactate	N/A	N/A	N/A	3.8
1.5	*Shewanella oneidensis* MR-1	Gold	Lactate	Ferricyanide	0.6	16.3	130

The first real breakthroughs in terms of increased power densities came upon the use of using air cathodes in micro-MFCs. Power densities of 73 W m^{-3} and 115 W m^{-3} were reported in works by B. E. Logan *et al.* in 2007.[93, 94] These were achieved using open air cathodes in normal-scale MFCs, but were a significant improvement on MFCs using other cathode materials. In 2006, a mini-MFC using carbon felt electrodes obtained a higher power density than this, using a ferricyanide catholyte.[95] However, more recently, Fan *et al.* managed to increase the power density to 1010 W m^{-3}, using carbon cloth electrodes in a mini-MFC with an air cathode in 2007.[91, 96]

It has been demonstrated that single chambered miniature MFCs can suffer from oxygen crossover from the cathode to the anode. This effect reduces the power output of the devices, as the small electrode separation means oxygen can diffuse from the cathode to the anode, and combine with electrons there. This effect was investigated by Logan *et al.* who found that the thickness of porous electrodes such as carbon felt could be increased to help better maintain anaerobic conditions at the anode.[97]

However, work by Mink *et al.* demonstrates that membraneless miniature MFCs, with an air cathode could still be suitable for power generation, despite the lack of membrane. However, the membrane helps to keep air from penetrating the chamber where it can reduce the power output of the device by combining with electrons. Therefore, they achieved the highest power densities using porous electrode materials, which helped to shield the biofilm from the oxygen which penetrated the system.[98]

As shown by the above results, although the chamber volume has a significant effect on the power density of an MFC, other factors such as the catholyte and anode material can have an even greater effect – some of the greatest power outputs come from devices which are bigger, but use an open air cathode. Since the initial leap by Fan *et al.* producing mini-MFCs with high power densities, it has proven difficult to make further advances, and as yet, there are only limited published attempts to consolidate findings from MFCs of the same chamber volume against other factors such as catholyte and anode material.[91]

It has been observed that scaling up these miniature devices leads to decrease in power density output, due to larger internal resistance in the devices arising from mass transport issues.[91] Furthermore, upscaling can create issues with large investment costs (mostly down to expensive electrode materials) and large scale MFCs suffer technical issues relating to flow effects across large surfaces and resultant poor electron transfer.[99]

It has been suggested that rather than further attempt to increase the power output of MFCs, they could instead be further miniaturised and "numbered-up" rather than scaled up. This would mean that rather than making larger devices with greater internal resistance, increased power density could be obtained through making miniature devices and linking them together. [100] This approach is rapidly gaining attention in research, since its inception in 2009. Woodward *et al.* discovered that operating two MFCs in tandem yielded a higher power output when they were electronically connected. They used a mathematical algorithm to further optimise their performance based on factors such as external resistance.[101]

Several examples since this time have shown that the electrical connection of multiple devices or "stacking" leads to increased power output of the MFCs. [100] Recently, Chouler *et al.* reported over a 10 times increase in the power output of miniature μL-scale MFCs (powered by artificial urine) when three were connected in parallel.[12] Furthermore, they reported that a 2 times increased electrode length led to an order of magnitude higher power density, when electrode separation was kept constant. This effect was due to

increased mass transport due to larger surface area, whilst maintaining low internal resistance due to small electrode separation. Similar work on mL-scale MFCs by Ieropoulos *et al.* demonstrated the same effect, on a similar substrate (urine), reporting around a 2 times increase in power when 48 of these devices were connected in parallel. [102] This demonstrates the effect of further miniaturisation on power density.

Miniaturisation of MFCs also facilitates their portability, enhancing their ability to be used for applications such as powering electronic devices or sensors. Work by Zuo *et al.* has demonstrated portable paper-shaped miniature MFCs for single-use sensing applications to detect certain species of bacteria.[103] Further work by Ieropoulos *et al.* has even demonstrated miniature (mL-scale) MFCs for use in powering a commercially available mobile phone.[104]

To even further miniaturise MFCs, several studies have developed microfabrication techniques, which manufacture micro-scale MFCs using microfabrication techniques. These devices were highly efficient and gave large power densities, using microfluidic delivery of nutrients.[15]

A systematic study of chamber sizes in these microfabricated devices revealed that the smaller the chamber volume was, the higher power density was achieved, implying that further miniaturisation is still worth exploring. This study by Qian *et al.* has achieved 62.5 W m^{-3} with only a 4 µL chamber size.[105] This is not the highest ever performance of a miniature MFC, but was much greater than larger scale fuel cells made using the same materials.

Given that at present the power densities achievable by MFCs has reached a plateau, much focus has turned to exploring the potential uses of mini- and micro-MFCs for use as electrochemical biosensors, particularly in the area of environmental chemistry.

3.0 – LITERATURE REVIEW

3.1 – Microbial Fuel Cells as Biosensors

The major advantage of MFCs as biosensors is that they offer the potential to be used *in-situ* and online. Since the current generated by an MFC directly relates to the metabolic activity of the bacteria in the biofilm at the anode, any disturbance of their metabolic pathway results in a change to the electrical output of the device.[1, 11] Provided that external factors and operating parameters such as temperature, pH and the conductivity and concentration of the feed solution are kept constant, these changes in current can be attributed to the specific disturbance applied. [70, 106]

In the case of an MFC, the anodic biofilm acts as the bioreceptor, recognising the presence of a toxicant, and this disturbance affects the rate of flow of electrons from the bacteria to the anode (which acts as an internal transducer), producing a detectable current change. This negates the need for an external transducer, giving them a distinct advantage over other types of biosensor. [1] Furthermore, they do not rely on an externally applied voltage, as the driving potential is provided by the oxidation of fuel on the anode and the reduction of an oxidant at the cathode.[11] Their ability to provide their own power, by oxidising a fuel source *in-situ* makes them particularly suitable for monitoring in more remote locations or long-term environmental monitoring. [56, 65]

3.1.1 – Heavy Metals

As MFCs developed, they became increasingly promising for use in applications such as wastewater treatment, coupled with power generation or manufacture of a useful product (*e.g.* hydrogen). During the process of the development of this technology from lab-bench scale to larger-scale trials, some of the potential problems which could arise for long-term use of these devices for this application were considered. The effect of toxins on the performance of these devices was not known – as such, it was identified as a potential barrier. [107]

When operating under fuel-saturated conditions, when all environmental factors are controlled, it has been demonstrated that a sudden change in the current output of an MFC can be attributed to the presence of a bioactive molecule in the feed stream. This allows MFCs to be used as biosensors for toxic compounds in wastewater. [1, 11]

In 2010, Schröder *et al*. set out to investigate the effects of toxins on the current output of MFCs.[107] They tested a range of toxins, including Cu^{2+}, Ag^{+}, Hg^{2+} and Pb^{2+}, as well as some

antibiotics. The toxins were used at concentrations roughly approximate to those found in well-treated wastewater and toxins were injected into the cells for periods of between 12 hours and 2 days, at steadily increasing concentrations. The authors predicted the MFCs would be affected by the toxins, and their current output may drop.

Their results reported that during such experiments, no significant drop in current output was produced. The authors attributed this to a well-reported effect in medicine – that biofilms are hard to treat with anti-microbials, as they have a high natural resistance to toxins. This effect is often reported in treatment of patients with various conditions in medical literature.

However, it directly contradicted earlier work by Kim *et al*. which had reported a detectable and significant drop in current output caused by heavy metal toxicity.[108] This difference is likely due to difference in effect of different toxins, as well as the nature of the experiment. Low dose over a long time period may cause a rise in bacterial resistance to the toxins. Kim had used a higher dose over a shorter time period, whereas Schröder used a low dose, with slow incremental increases to larger concentrations. Schröder concluded more research was needed to develop a greater understanding of such effects, and those of different anti-microbial compounds.

Kim *et al*. had also developed a system by which two MFCs could be used during measurements of real wastewater – one acting as a reserve sensor, while the other was actively involved in the sensing of toxic components, should the biofilm be damaged. This approach means sensing can be resumed more quickly after a toxic event. They used this sensor to detect Pb and Hg, as well as the organic molecule PCB. They recorded a cumulative effect when the toxins were used in conjunction, however the sensors were unable to qualitatively analyse which toxin was which.[14] This, however is not too dissimilar from some currently-used methods such as fish and *Daphnia.*

Since then, the work of Stein *et al.* has proven the use of MFCs for detection of nickel[4] and the work of Di Lorenzo *et al.* has demonstrated their use for the detection of cadmium, seemingly disproving these findings.[1] Crucially, both these works used MFCs as on-line, continuous sensors for the toxic metals, rather than “shock”-sensors, which detect only a large toxic event. This makes them more useful to the water treatment process, as results can be obtained in a matter of minutes, rather than taking samples periodically.

Jiang *et al.* also detected Cu^{2+} ions using MFCs with standard electrodes in a continuous flow system, and with a similar setup, using flow through electrodes (rather than flow-by electrodes used in the previous setup). They reported that sensitivity could be increased between 15 and 41 times depending on the flow parameters of both set ups. They also reported better sensitivity when the anode potential was controlled with a potentiostat, anywhere between -0.41 V and +0.1 V, but that electrodeposition of copper biased the toxicity measurement at lower potentials. The optimum value was therefore found to be -0.15 V, giving the highest sensitivity possible, without any bias.[16] The effect of potentiostatic control was also noted by Stein *et al.* who did not report a problem with electrodeposition of copper, but noted that the magnitude of change in current was greatest at - 0.4 V. [13] Other work by Stein *et al.* found that the same potential was also optimum for the detection of nickel. However, the overpotential at low concentrations of Nickel was greater than that at high concentrations, causing a bigger response to low concentrations of nickel than high concentrations of nickel. The authors also noted that the sensitivity towards nickel was not great enough to detect concentrations found in drinking water, and that more work needed to be done on improving the sensitivity. [4]

Other work by Stein *et al.* has demonstrated how kinetic control can be achieved to tune the sensor to detect specific toxins, and work by Feng and Harper has also researched the use of artificial neural networks to better process and understand the signals we receive from MFCs in order to elucidate the nature of the toxicant responsible.[4, 75, 83, 109] Both works are critical to making useful biosensors, which can discriminate between different toxicants.

3.1.2 – Organic Pollutants

One major method of detecting the presence of organic molecules in wastewater is to measure biochemical oxygen demand (BOD). Although this method does not provide identification of the compounds in question, it gives an indication of a failure to remove the organics during the treatment process. It only measures those compounds which are biologically active (i.e. being oxidised by organisms). Traditional test methods are slow and can take up to 5 days to complete.

Provided the anodic reactions within the MFC are the limiting step, under non-saturated fuel conditions, an alteration of the concentration of biodegradable organic matter fed to the system will result in a direct change in the amount of electrons transferred to the anode, leading to a change in the output current of the device.[1, 3, 11] As such, this allows MFCs to be used as a biosensor for the monitoring of BOD in wastewater.

In 2003, Kim *et al*. reported the design of a BOD sensor, based upon on an MFC; a schematic diagram is shown in Figure 10.

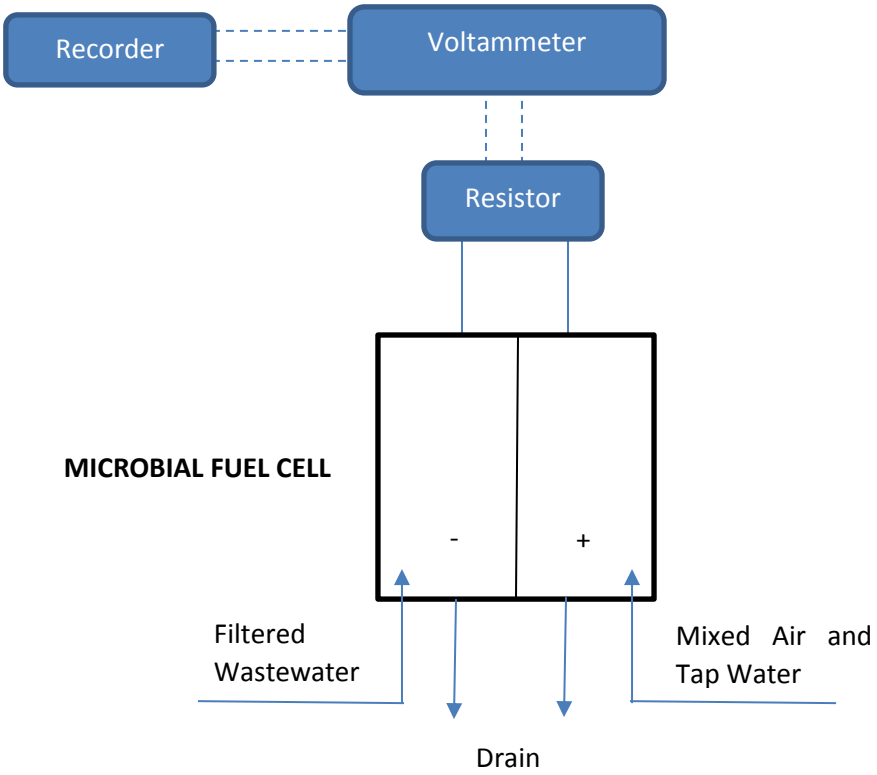

Figure 10 – Schematic Diagram of a BOD sensor (adapted) [110]

The Coulombic yield was shown to correlate to the BOD (measured using the standard BOD_5 test) in a linear fashion with synthetic wastewater. The sensor was then used on-site at a wastewater treatment plant for 60 days. The sampling period was only 45 mins rather than 5 days for a BOD_5 test, which is a great improvement. The sensor was found to be stable and accurate over this period, and the linear relationship was maintained with real wastewater. However, longer lab-based tests showed the system needed continual recalibration for long-term use. Furthermore, air and clean water needed to constantly be supplied to the MFC to ensure its continual operation.[110] Despite these drawbacks, this work proved the basic principle as viable.

In this experiment, the MFC had two separate chambers of 17 mL each. Both electrodes were carbon felt, and the chambers were separated by a proton exchange membrane (PEM) of Nafion 424.

Since then, Kim has enhanced this technology, by reducing the size of the MFC to reduce internal resistance (detailed in Section 3.3) and has reported improved performance of an almost identical sensor based on an air-cathode. An air-cathode consists of the electrode

material (in this case carbon felt) to which the PEM has been directly fused, effectively forming a one-chamber fuel cell. The advantage of this is that the air-cathode can be placed in the open and draw oxygen directly from the air, rather than needing it to be continuously fed to the system. Conditions of hot-pressing the membrane and cathode together were found to be important in terms of creating a well-performing electrode. The optimum was found to be at 120 °C, with 150 kg cm^{-2} of pressure for 30 s. When air-cathodes were applied to the same MFC BOD sensor, sensitivity increased from 1.2×10^{-3} to 1.8×10^{-2} C L mg^{-1} – almost a 10-fold improvement. However, it was reported that excess liquid in the cathode during long periods of operation posed a problem. Although some is needed for proper hydration of the membrane, too much was found to limit oxygen diffusion to the cathode. [77, 108]

Also in 2003, Kang *et al.* reported the use of MFCs for low BOD measurement. Their design used graphite electrodes, which were platinum coated in order to increase catalytic activity at both electrodes, but also to increase oxygen affinity at the cathode, to prevent oxygen diffusion to the anode. To further prevent this diffusion, which limits performance of the device, they decreased the size of membrane used, limiting diffusion. Both the catalysis and the reduced membrane size were found to increase the performance of the MFCs. [111]

Di Lorenzo *et al*. reported in 2009 use of a single-chambered MFC with a chamber volume of 50 mL with a carbon cloth anode and an air-cathode comprised of carbon paper, coated with a carbon-supported platinum catalyst. The device was found to have high reproducibility and stability over its 7 month operating period. Once again, it was found that there was a linear relationship between Coulombic output and BOD value, up to 350 mg cm^{-3}. The response time of this system was in the range of hours (depending on factors such as external resistance), but this was greatly reduced by a 75% reduction in chamber volume (to 12.6 mL), to a value of only a few minutes. This was attributed to increased Coulombic efficiency (9 times greater) and reduced retention time. This sensor was also tested upon real wastewater, and a good correlation between BOD and Coulombic output was maintained.[37]

In 2011, Nogueira *et al*. reported a similar set of results, this time linking them to several variables such as temperature, conductivity and pH. The same linearity was observed between BOD and output from the fuel cell when these variables were controlled, but was seen to vary when these conditions were altered. It was deemed necessary to apply a correction factor when processing these results to obtain accurate values of BOD.[78] Work by

Yang also confirmed the relationship between pH and device performance, also studying effects of organic matter concentration and retention time on the response time of the device.[112]

Also in 2011, Zhang and Angelidaki developed a submersible MFC to monitor BOD in groundwater. The submersible MFC comprises of a single chamber and can be used *in-situ.* It requires a feed of air into the cathode to act as an oxidant. The current output was found to have a linear relationship with BOD content of the wastewater, up to 250 mg L^{-1}, with a response time of less than 0.67 hours. The submersible nature of the sensor makes it particularly suited to in-field measurements.[113]

In 2014, Di Lorenzo *et al*. built on earlier work, designing a fuel cell with a chamber volume of only 2 mL, recording a response time of only 2.8 mins – much more applicable to on-line, real-time sensing of wastewaters than previous work. However, this sensor monitored chemical oxygen demand (COD) rather than BOD. COD measures total concentration of chemically oxidisable species in a sample, rather than just those which are biologically active. The linear detection range was 3–164 ppm, with a sensitivity of 0.05 μA mM^{-1} cm^{-2} with respect to the anode total surface area. The electrodes were both of carbon cloth, the air-cathode being hot pressed with Nafion 141.[1] Feng *et al*. have also shown demonstrated the use of MFCs as COD sensors, suggesting that COD actually correlates better to area under the peak on a plot of current *vs.* time than the peak height. Furthermore, they made innovative use of artificial neutral networks to interpret the electrical signals from the MFC more accurately, across a wide range of concentrations, with synthetic and real wastewater.[109]

In 2007, Kumlanghan *et al*. reported a biosensor based on a single-chamber MFC which detected the presence of glucose in artificial wastewater. The response of the sensor was linear up to 25 g L^{-1}, and the lower detection limit was found to be 0.025 g L^{-1}. The response time was found to be between 3 and 5 mins. The sensor used a mixed bacterial culture from anaerobic sludge. Based on these results, he suggested that MFCs may be suitable for detecting any easily-degradable organic matter, and theorised that the mixed culture would be able to degrade other types of organic matter in a similar fashion to glucose (implying a single culture may have limited capability).[81] This sensor was not based on toxicity, but on the linear response of MFC output to the rate of oxidation of an organic feed source.

Work by Davila *et al.* has shown that MFCs with chamber volumes as low as 144 μL can be "numbered-up" rather than scaled up to produce working biosensors that are capable of

detecting the presence of formaldehyde. The maximum concentration used was 4 % (w/w) in artificial wastewater and the minimum detectable was 0.1 % (w/w). However, all doses caused irreversible inactivation of the biofilm, meaning a new one must be grown with each use. However, response time was so low, it was deemed to be "immediate".[15] This work proved the concept of MFCs as sensors for organic, toxic compounds, disproving earlier work by Schröder *et al*. who had observed no effect of both metal and non-metal toxins on a biofilm of the same composition (Geobacter), but highlighted that further work was needed to understand the effects of different toxins and different biofilms. [15, 107] Work by Zhou *et al*. confirmed the susceptibility of biofilms to formaldehyde in lower concentrations, between 0.01-0.10 % (w/w) and did not observe complete inactivation of the biofilm at concentrations other than 0.10 %. They were able to distinguish between different concentrations when taking into account the baseline current and toxicity of the different doses on their single-culture biofilm.[10] Wang *et al.* also monitored formaldehyde using an MFC with a single bacterial culture (*S. Oneidensis*) and could detect from 0.01 to 0.10 % (w/w) formaldehyde without permanent damage to the biofilm.[10]

Work by Stein *et al*. demonstrated the detection of SDS – a common organic surfactant found in many cleaning products, and therefore wastewater. This work is also one of the earliest into the detection of an environmentally-relevant organic molecule, and concluded it could be detected with high sensitivity.[75]

In 2014, work by Liu *et al.* demonstrated the ability of batch-mode MFCs to detect sodium acetate in artificial wastewater. Acetate is well-known as a fuel source for MFCs. The response to the shock acetate does (of 200 mg L^{-1}) was positive, confirming the relationship between metabolisation of the fuel source and voltage output. This work also investigated metal toxicants (Cr^{6+} and Fe^{3+}) and a common nutrient, nitrate (NO_3^-). As expected, the metals were both toxic, and caused negative responses. The greater negative response was produced by the more toxic metal, Cr^{6+}, and the response was proportional to the concentration of the shock. The nitrate shocks exhibited no clear voltage change. A mixed toxic event containing both sodium acetate and chromium in varying ratios was also investigated. These events always caused a sharp voltage drop within 30 mins, but were sometimes accompanied by initial voltage increases. This highlights the complications with investigating real-life toxic events in real wastewater – the combined effect of multiple compounds is difficult to elucidate. [17]

3.1.3 – Detection of Other Toxicants using MFCs

Other toxicants have been shown to affect MFCs in similar ways, and have successfully been detected in similar experiments. Shen *et al.* have demonstrated the ability of MFCs to detect the presence of HCl in artificial wastewater. They reported that as retention time in the fuel cells was decreased, sensitivity to the toxin increased substantially. Moreover, the extent of inhibition of current generation was found to be related to the dosage, suggestion a dosage-response relationship exists.[5] They also reported that lower external resistances caused increased sensitivity to the toxic event, backing up earlier findings by Stein *et al*.

In 2007, Kim *et al.* reported the successful detection of organophosphorus compounds using an MFC with a mixed bacterial culture from anaerobic sludge. Organophosphorus compounds are used as pesticides, so this work would provide an insight into the effects of agricultural run-off in rivers. They found rapid monitoring, on a time scale of minutes, was possible to achieve a 61% signal ratio – a very high toxicity towards the biofilm at 1 mg L^{-1} concentration.[14] The toxicant in question was diazinon, a common insecticide. The same sensors were also found to detect chlorinated organic compounds, known as PCBs (polychlorinated biphenyls) which were once used as coolant fluids.

In 2010, Patil *et al.* reported the attempted detection of the antibiotic compounds sulfamethaxozole, sulfadiazine and chloramine B, but as with their results on metal toxicants, they found no significant response to the toxic events reported.[107]

Thus far, there has been no further attempt to observe compounds of a similar structure – either substituted or non-substituted aromatic compounds, similar to pesticides, pharmaceuticals, and anti-biotic compounds. Therefore, more work in this area is needed to confirm whether or not the work by Patil *et al.* is correct with these types of compound. However, work by Zhang *et al.* did use MFCs as a system to enhance the degradation of paracetamol and thus remove it from wastewater. Electrons were produced from the degradation of a fuel source in the anode chamber. These electrons were transferred to the cathode chamber, where they generated OH-radicals which were instrumental in the breakdown of paracetamol *via* a Fenton reaction. This is advantageous because the bio-electrons mean no replenishment of Fenton reagents is necessary, as they are regenerated by the electrons. [18] The presence of paracetamol was not directly monitored, because it was not present at the anode. However, in a single chamber system it may have been detectable, or it could perhaps be detected by a change in voltage output of the device as a single dose of paracetamol is given, however this was not investigated by the authors.

3.2 – Microbial Fuel Cells (MFCs) in Wastewater Treatment

Recently, MFCs have been proposed as a technology to combine the treatment of wastewater with the production of energy using a single device. The technology functions by using organic carbon in the medium being treated as a fuel source. It generates a current by metabolising that carbon to produce electrons. These electrons are then passed around an external circuit under load, in the usual manner. The organic matter in the treatment medium is metabolised and broken down and current is produced simultaneously.

MFCs are seen as holding advantages over other technologies due to their ability to treat low-concentration substrates at temperatures below 20 °C, where techniques such as anaerobic digestion fail, due to the cold conditions. [99]

However, the technology also experiences limiting factors which affect its scale up. One is the cost of expensive electrode materials, the others are performance-related. The main performance-related factors are the activity of biocatalysts at the anode, electron transfer between the bacteria and the anode, internal resistance arising from mass transfer between the electrodes and the overpotentials of both electrodes.

Of these factors, the activity of the biocatalysts is thought to be hardest to control, as the microbial communities and microbial activity in MFCs are not well understood. Although it is commonly regarded that biofilms are a common structure of microbial communities in MFCs and that within these biofilms, facultative anaerobes are usually found within electrochemically active consortia, it is not known exactly how these bacteria arrange themselves, and their electron transfer pathways are not yet fully known, especially in examples with mixed bacterial species. [99]

The mechanism of bacterial electron transfer to the anode is somewhat better studied. There are two main hypotheses describing how this could occur. One describes a direct electron transfer between an outer-membrane protein and the electrode surface. It is suggested that cytochromes may be a candidate for such a transfer.[114-117] The other hypothesis describes a system in which the electrons are transferred to the electrode via external or self-produced mediators. [99] Some bacteria have been observed to form nanowires, connecting them to the electrode surface and providing a path along which electrons can be conducted. [11, 118] More work is needed to understand which of these methods is correct, or if the answer is a combination of some or all of them. Whichever is correct, improving electron transfer at the anode surface would greatly help to increase the performance of MFCs.

Internal resistance is also considered a limiting factor in this technology. A high internal resistance causes considerable potential drop due to ohmic losses. This is true in MFCs with and without membranes. Decreasing the distance between the electrodes reduces this resistance, so careful design of MFCs can help to overcome this problem. [99, 119, 120]

The cathode reaction is considered to be the key limiting factor of applying this technology on an industrial scale due to its limiting effect on the performance of MFCs.[111, 120, 121] Often, oxygen is the cathodic electron acceptor, but there is usually poor contact between gaseous oxygen and the cathode itself, limiting the turnover rate. Moreover, oxygen leaking to the anode can occur, and its presence there reduces the efficiency of electricity generation, via combination of the oxygen with electrons. Catalysts such as platinum or bio-derived catalysts have been shown to help overcome this problem. [11, 99]

Some MFCs have been developed which can generate energy from domestic wastewater directly. For example, Min and Logan reported an MFC-based technology which could obtain 79% COD (Chemical Oxygen Demand - the amount of oxygen which is needed for the oxidation of all organic substances in water) removal, and generate an average power density with a 4 hour retention time using domestic wastewater.[122] This compares to a 68% COD removal using anaerobic digestion alone.[99] These devices generated a power density of 72 mW m^{-2}.

Other work by Logan and Liu reported an MFC design with a PEM membrane which could generate up to 28 mW m^{-2} power density at a 28% Coulombic efficiency (the efficiency with which charge (electrons) is transferred in a system facilitating an electrochemical reaction). When the PEM was removed, the power density produced was 146 mW m^{-2}. This indicates that in some cases the PEM can inhibit the performance of an MFC. This is replicated using other fuel sources such as single carbohydrates. The COD removal was found to be 55% with the PEM, but 75% without. [119]

Similar work by Min and Angelidaki developed this technology to make the devices submersible, for in-flow use, and could generate power densities of up to 244 mW m^{-2}. This made them more industrially relevant as the devices can be used in conditions more applicable to a water treatment environment, and do not require a specific chamber for the anode, as the medium acts as this chamber. [123]

Other devices have concentrated on generating energy from more specific kinds of waste such as urine. The rationale behind these devices is that they could be used directly on urine

at events such as music festivals or in large buildings to immediately treat waste urine and generate electricity. Work by Chouler *et al* reported a device which could generate up to 0.580 W m^{-3} using an artificial urine medium, and that the power output was over ten times higher when three devices were connected in parallel.[12] The authors further reported that a biomass-derived catalyst improved the power density to 1.95 W m^{-3}. The removal of the artificial urine components was not studied.

Work on using MFCs to treat real urine waste has been carried out by Ieropoulos *et al.* who found they could achieve power densities of up to 4.93 mW m^2 when 48 miniature devices were connected in a stack and fed with urine. The authors reported a conversion efficiency of >50% for each device type tested in the study.[102]

Another waste type which has been cleaned by MFCs is swine manure. Work by Vilajeliu-Pons *et al.* has demonstrated that MFCs can be used to clean up swine manure. This is usually done by anaerobic digestion, but this does not reduce the nitrogen content. The MFC-based technique reported a removal rate of up to 2.09 COD m^{-3} day^{-1} with the added benefit of a nitrogen removal rate of up to 0.16 kg N m^{-3} day^{-1}. The max power density obtained was 2 mW m^{-3}. [124]

This shows the possibility for MFCs to be used in the treatment of a wide variety of different waste types, in some cases with better COD removal rates, with added benefits of removing more compounds, in more facile conditions, and with the added economic bonus of power generation.

3.3 – Control of Anode Potential

A problem often encountered in this field of work is that results obtained simultaneously from identical MFCs were not always comparable as the baseline currents of different MFCs were not always the same. This could be due to a variety of factors, such as uneven biofilm growth at the anode, small differences in flow rate as blockages occur and then break apart, differences in biofilm composition, etc.

One method of controlling this is to control the anode potential of each cell, to ensure the same environment is in every MFC, to help ensure more reliable biofilm growth. It was decided to explore if this technique could help results which were more comparable.

Work by other groups has shown that control of anode potential can provide a more stable baseline current, which is crucial for toxicant detection, as it makes results from different

fuel cells more comparable. [13] It is through the control of overpotential that this stability arises. Anodic overpotential is defined in Equation (5).

$$\eta = E_{an} - E_{electron\ donor} \quad (5)$$

Where η is the anodic overpotential (V), E_{an} is the anode potential (V), and $E_{electron\ donor}$ is the chemical potential of the fuel source under the present conditions. The reaction taking place at the anode is:

$$C_2H_3O_2^- + 4\ H_2O \rightarrow 2\ HCO_3^- + 9\ H^+ + 8\ e^-$$

Therefore, the chemical potential of the fuel source can be calculated using Equation (6), which is derived from the Nernst Equation. [13, 92]

$$E_{electron\ donor} = E_0 + \frac{RT}{nF} \ln \frac{[HCO_3^-]^2[H^+]^9}{[\text{Acetate}]} \quad (6)$$

Where E_0 is the standard reduction potential of the half reaction (V), R is the gas constant (8.314 J mol^{-1} K^{-1}), T is the temperature (K), n is the number of electrons involved in the half reaction, and F is Faraday's constant (96485.3 C mol^{-1}). In the case of acetate, E_0 is +0.187 V *vs.* NHE.

Since the amount of energy released per electron is heavily dependent on the overpotential, and the rate of reaction also increases within certain limits, it is necessary to control the overpotential to keep the baseline current stable. [13, 125-127] At low anode potential, microorganisms gain little energy and will produce a low current. When the anode potential is too low, below $E_{electron\ donor}$, microorganisms will gain more energy from anaerobic fermentation than from electron donation to the anode and they will switch to this different metabolism if the substrate allows fermentation. As such, no current can then be observed and the presence of toxic compounds cannot be detected. The anode potential thus needs to be controlled at a potential higher than $E_{electron\ donor}$. [13, 72]

4.0 – METHODOLOGY

4.1 – Materials

All reagents were of analytical grade, and used as purchased, without further purification. They were provided by Sigma-Aldrich, with the exception of iron (III) chloride and potassium acetate, which were purchased from Alfa Aesar (Heysham, UK). Deionised water was made on site by reverse osmosis. The micropollutants diclofenac, bisphenol-A (BPA) and 17β-estradiol were purchased from Sigma-Aldrich (Gillingham, UK).

Nafion 117 Proton Exchange Membrane (PEM) was purchased from Sigma-Aldrich (Gillingham, UK). Titanium wire was purchased from Advent Research Materials (Witney, UK) and stainless steel mesh from Inoxia Ltd. (Cranleigh, UK)

Internal standards for LCMS, bisphenol-A-D16 and 17β-estradiol (2,4,16,16-D4) were purchased from Sigma-Aldrich (Gillingham, UK) and LGC standards (Middlesex, UK). For the preparation of mobile phases, HPLC grade methanol (MeOH) and ammonium fluoride (NH_4F) was purchased from Sigma-Aldrich. Water (H_2O) was of 18.2 MΩ quality (Elga, Marlow, UK).

4.2 – Fuel Cell Design

For preliminary experiments, the design of the cells used was different to the cells which were eventually used. These fuel cells were constructed as in Figure 11. Each layer of the fuel cell was 3D-printed and a silicone rubber gasket was placed between each. The anode was of carbon cloth.

Three different types of cathode were constructed. The first was made of carbon cloth. Nafion 117, was used as a PEM membrane, which was hot pressed to the cathode for 15 minutes under 1480 kg m^{-2} pressure, at 180 °C.

The second cathode consisted of carbon cloth coated in conductive carbon paint, annealed for 2 hours at 120 °C. The third type of cathode was similar, but a stainless steel mesh was adhered to the carbon cloth using a layer of the carbon paint, and the resulting electrode annealed under the same conditions.

In all cases, and the electrode dimensions were 13 x 24 mm. Titanium wire was used as the electrical contact for the electrodes.

The third electrode design was taken forward for use, as it demonstrated the best electrical contact and least leaking.

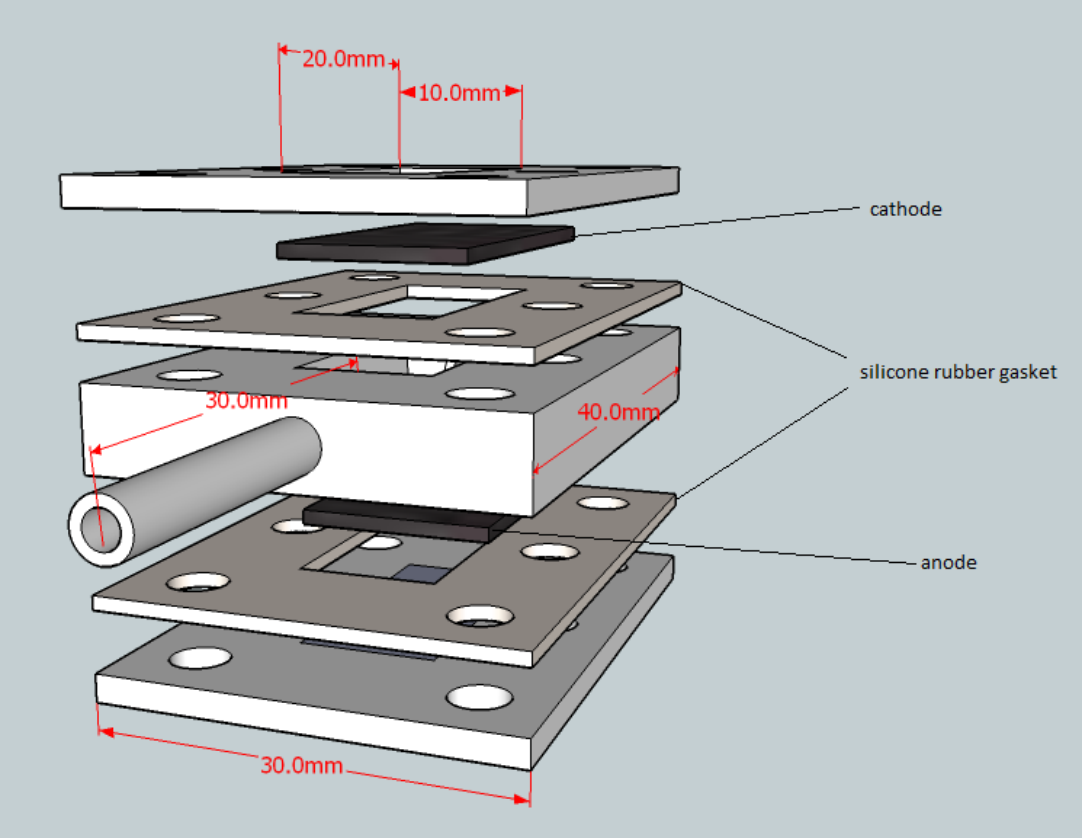

Figure 11 – Schematic Diagram of MFCs Used for Preliminary Micropollutant Injections.

The materials were assembled as above, and stainless steel bolts (4 mm diameter) were inserted through the holes in the body of the fuel cell and tightened, before a layer of silicon sealant was applied to the outside edges to ensure the cells were watertight. The MFCs were inoculated as per the methodology, and were then connected to a 1 kΩ external resistor.

However, these MFCs were found to be prone to leaking and were discontinued from use after the initial preliminary experiments.

For further experiments, a different design was used. They consisted of a single chamber made of a rectangular piece of PDMS sandwiched between two Perspex plates. A mould to cast the channel and chamber in PDMS was made of PA 2200 nylon plastic and purchased from Shapeways (The Netherlands). See Figure 6.

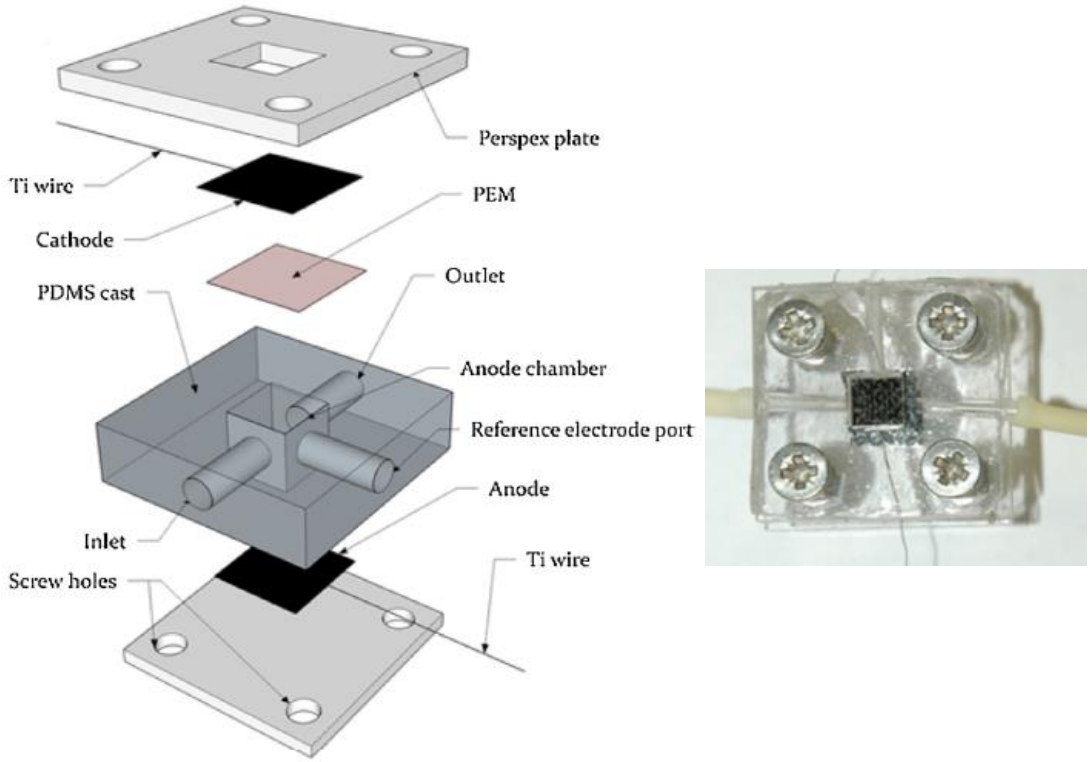

Figure 12 - Schematic Diagram (left) and photograph (right) of MFCs used in subsequent investigations [12]

The PDMS (Dow Corning Sylgard 184, Ellsworth Adhesives, UK) was mixed together from two solutions – a crosslinking agent and the pre-polymerised PDMS in a 20:1 ratio, and air blasted to remove bubbles, before being tipped over the cell mould and cured in an oven at 60 °C for 1 hour.

Both electrodes were produced from carbon cloth, with titanium wire threaded through them for electrical contact. The cathode was also threaded to a very fine stainless steel mesh, and hot-pressed to Nafion 115 Membrane under approx. 2.5 bar pressure at 150 °C for 12 mins. The total chamber volume was 64 µL (4 x 4 x 4 mm) and the surface area of both electrodes was 16 mm^2.

4.3 – Fuel Cell Startup and Operation

Artificial wastewater (AW) was used as fuel, containing potassium acetate (0.071 M) as a carbon-based energy source. AW was produced by adding the following salts to deionised water: (NH4)2SO4 (2.040 mM), MgSO4.7H2O (0.243 mM), MnSO4.H2O (0.0355 mM), NaHCO3 (1.550 mM), FeCl3.6H2O (0.011 mM) and MgCl2.2H2O (0.032 mM). This fuel was autoclaved prior to use.

Fuel was flowed into the MFC using an IsmaTec BVK MS-CA 8/6 peristaltic pump, with a flow rate of 1.4 mL min^{-1}.

After inoculation with bacteria from anaerobic sludge (donated by Wessex Water, 1 mL per 100 mL of feed), the cells were connected to a Pico Technology High Resolution Pico Datalogger to monitor the cell voltage. After 1 hour, the cells were connected to an external resistor of 15 kΩ. When the output voltage was stable (after at least 3 days) experiments could begin.

4.4 – Electrochemical Characterisation of MFCs

Electrochemical Characterisation of the MFCs was carried out using a PGSTAT128N potentiostat, (Metrohm, UK).

Cyclic voltammetry was carried out using Ag/AgCl reference electrodes supplied by Greenleaf Scientific Ltd. (Ireland), which were inserted into the chamber within the MFC. The counter electrode (CE) and working electrode (WE) were the electrodes in the MFCs; the anode as the WE and the cathode as the CE. Cyclic voltammograms were obtained with 3 sweeps, between -0.4 and +0.4 V and with a scan rate of 10 mV s^{-1}. The values for scan rate and potential window were consistent with literature. [72, 128, 129] Initially, CVs were performed with a smaller potential window, which was gradually widened to ensure these potentials would not damage the biofilm, but it was found that they were suitable. The three-electrode system was also consistent with literature, although a very small Ag/Cl reference electrode was selected (rather than a large SHE), due to the miniature nature of the MFCs in this investigation. The potential of the anode will change as biofilm develops on it, and thus it is necessary to use a reference electrode for electrochemical characterisation of the electrode, to ensure the applied voltage is exactly the same each time. [72]

Polarisation curves were obtained using an external resistance box, connected in place of the fixed resistor, and were performed on 4 MFCs. The resistance was varied between 0 and 100000 kΩ. After each new resistance value was applied, a time of 30 minutes was allowed for the output voltage to stabilise, before altering the external resistance again.

For electrical impedance spectroscopy, the potentiostat was operated in Electrical Impedance Spectroscopy mode, with an additional FRA23M Impedance Analyser installed (Metrohm, UK). EIS was carried out between 0.1 Hz and 100 KHz, and was then mathematically analysed using the pre-installed software package. The equivalent circuit fitting used the circuit shown in Figure 13.

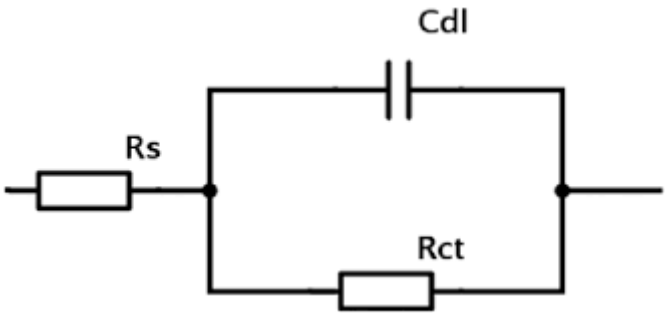

Figure 13 - The Equivalent Circuit used for Mathematical Fitting of EIS results - a simplified Randle's Circuit

C_{dl} is the double layer capacitance within the MFC, R_s is the resistance from the electrolyte, and R_{ct} is the charge transfer resistance. The values for both types of resistance were calculated in the experiment, to determine C_{dl}.

During electrochemical experiments, cells were fed the usual feed medium throughout.

4.5 – Injection of Micropollutants

MFCs were set up in accordance with the diagram in Figure 14 and operated in continuous mode.

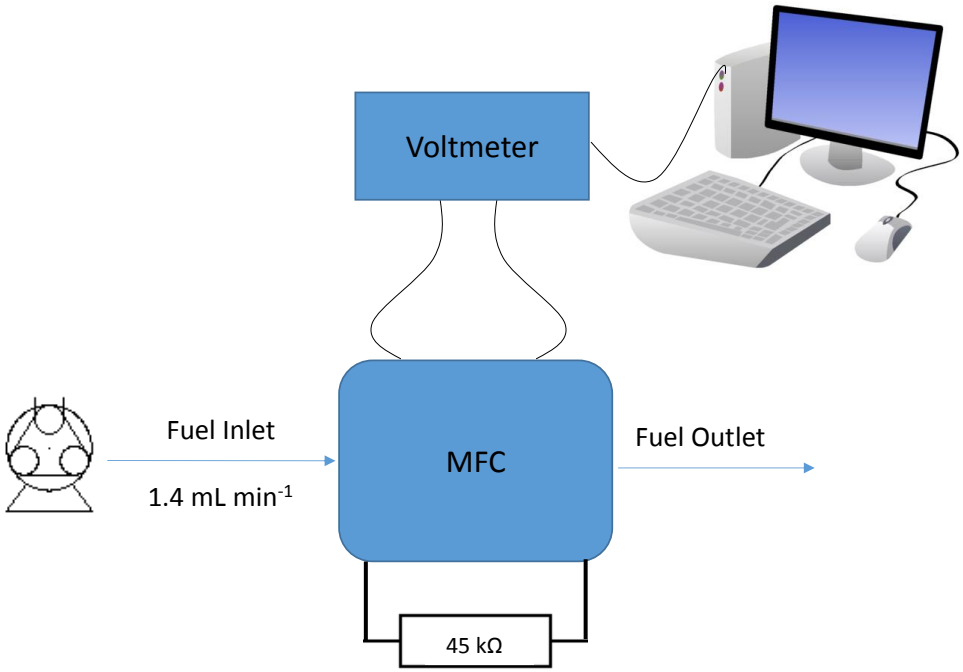

Figure 14 - Experimental Setup of the MFCs

Diclofenac, triclosan, bisphenol-a and 17β-estradiol were tested as bioactive compounds in this study. The injection was performed by feeding the MFCs for 30 minutes with the fuel containing the target compound. After the injection, the MFCs were fed with the fuel without micropollutant for at least 3 hours to allow recovery before a new test was performed.

The average baseline current before the toxic event (σI_0) was obtained by calculation from the output voltage, which was recorded. The output current 30 minutes after the injection of toxicant (I_t) was also calculated in the same way. These two values were used to calculate the signal ratio, as seen in Equation (11).

$$\text{Signal Ratio} = \frac{\sigma I_0 - I_t}{\sigma I_0} \qquad \text{Equation (11)}$$

The signal ratio provides a measure of the amplitude of the signal against the baseline operation of the fuel cell and therefore allowed MFCs to be compared.

The sensitivity of the MFC-based biosensors to each micropollutant was calculated using Equation (12):

$$\text{Sensitivity} = \frac{\Delta \partial I}{\Delta C_{\tau} \times \text{CSA}} \qquad \text{Equation (12)}$$

where ∂I is the change in current observed after injection of a micropollutant, C_{τ} is the concentration of micropollutant and CSA is the cathode surface area.

Samples were taken before and after each micropollutant injection for analysis by LCMS to elucidate how much of each compound had been metabolised.

4.6 – Long Term Exposure to Bioactive Compounds

The effect of long term exposure of the bioactive compounds to the MFCs was investigated to help provide some insight into two areas:

1) Long term exposure of MFCs to a micropollutant at a single concentration – does this lead to any change in behaviour of the biosensor, or any resistance to the micropollutant

2) Exposure of the MFCs to varying concentrations of micropollutant in order to mimic the response of the MFC to a change of concentration of micropollutant in real-life applications

In the first type of experiment, 3 MFCs were fed with clean feed overnight. After this, 2 MFCs were exposed to the target compound at 10 µg mL^{-1} for a period of 3 days. The third cell acted as a control. The voltage response of the MFCs was monitored over the period.

In the second type of experiment, 3 MFCs were again fed with clean feed overnight. After this, 2 were exposed to micropollutants, starting with low concentrations, and increasing to higher ones. The voltage response of the MFCs was monitored, and the concentration was increased whenever this output had reached a steady state. The third cell acted as a control. The total duration of the experiment was 3 days.

4.7 – Stacking Experiments

The purpose of these experiments was to understand the effect of linking multiple MFCs together, and in order to ascertain whether the micropollutant could be removed after running through a series of MFCs.

Four MFCs were fed with a clean solution overnight. One acted as a control throughout the experiment. After this, one was fed with the compound under investigation at 10 µg mL^{-1} for 24 hours, whilst the others remained on clean feed.

This cell was then connected to one of the other cells, and both were fed with micropollutant for 24 hours, then connected to the last cell, all fed with micropollutant for 24 hours (see Figure 15). The voltage output across each device, and the total voltage output of the stack was monitored. Samples were taken for LC-MS analysis at the inlet and after each MFC, before the next was added.

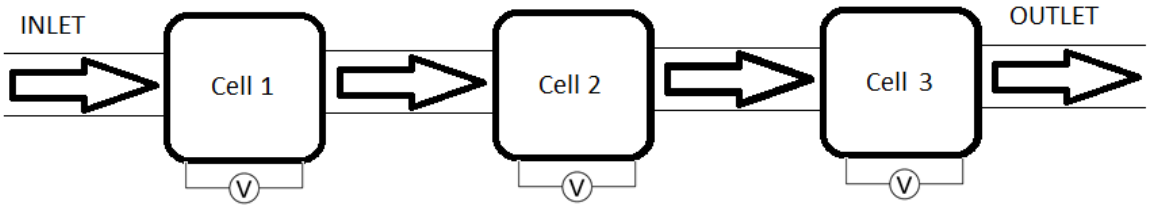

Figure 15 - Stack Experiment Setup

4.8 – LC-MS Investigation

Water samples were spiked with Bisphenol-A-D16 and 17β-estradiol-D4 to achieve a concentration of 100 ng mL^{-1}. These were then analysed using a fully validated ultra-high-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) method (Petrie *et al.*, 2016). Separation was achieved using a reversed-phase BEH C18 column (150 x 1.0 mm, 1.7 µm particle size) (Waters, Manchester, UK) with a 0.2 µm, 2.1 mm in-line column using a Waters Acquity UPLC system (Waters, Manchester, UK). A gradient of 1 mM NH_4F in 80:20 H_2O:MeOH (mobile phase A) and 1 mM NH_4F in 5:95 H_2O:MeOH (mobile phase B) was applied. Initial conditions of 100 % A were maintained for 0.5 min before reducing to 40 % over 2 min and further reduced to 0 % over 5.5 min. This was maintained for 6 min before returning to starting conditions for 8.4 min to re-equilibrate the column. The column

was maintained at 40 ºC and the mobile phase flow rate was 0.04 mL min^{-1}. The injection volume was 15 µL.

The UPLC system was coupled to a Xevo TQD Triple Quadrupole Mass Spectrometer (Waters, Manchester, UK), equipped with an electrospray ionisation (ESI) source. Analytes were analysed in negative ionisation mode (ESI -) with a capillary voltage of 3.20 kV. The source temperature was 150 °C whilst the desolvation temperature was 400 °C. A cone gas flow of 100 L $h^{-1}$and a desolvation gas flow of 550 L h^{-1} was used. The nebulising and desolvation gases were nitrogen, and the collision gas was argon. The optimised MS/MS conditions can be found in Table 6. A seventeen point calibration curve was prepared ranging in concentration from the instrument quantitation limit to 500 ng mL^{-1}. Calibration coefficients (r^2) were >0.997. Samples out with the linear range were diluted and re-analysed. Information on the instrument performance is available in Table 7.

Table 6 - Optimised MS/MS conditions for the analysis of Bisphenol-A and 17β-estradiol

Compound	*Retention time (min)*	*Precursor ion (m/z)*	*Product ion 1 (m/z)*	*CV (V)*	*CE (eV)*	*Product ion 1 (m/z)*	*CV (V)*	*CE (eV)*	*Ion ratio*
Bisphenol-A	8.96	227.3	212.1	40	22	132.7	40	25	1.60
Bisphenol-A-D16	8.87	241.1	223.1	40	20	-	-	-	-
17β-estradiol	9.74	271.1	183.0	60	40	144.9	60	45	1.04
17β-estradiol-D4	9.72	275.1	147.0	60	40	-	-	-	-

Key: CV, cone voltage; CE, collision energy

Table 7 - Instrument performance for the analysis of Bisphenol-A and 17β-estradiol in 18.2 MΩ quality H2O

Compound	*IDL (ng mL^{-1})*	*IQL (ng mL^{-1})*	*Intra-day accuracy[a] (%)*	*Inter-day accuracy[a] (%)*	*Intra-day precision[a] (%)*	*Inter-day precision[a] (%)*
Bisphenol-A	0.03	0.10	106.1	108.7	2.6	2.6
17β-estradiol	0.09	0.47	96.9	93.7	4.5	5.2

Key: IDL, instrument detection limit; IQL, instrument quantitation limit

[a]Determined using a concentration of 100 ng mL^{-1}

This method is in line with those previously published.[130]

4.9 – Scanning Electron Microscopy (SEM) Imaging of Biofilms

The biofilms grown on the anode were prepared for SEM imaging using the following protocol.

The anodes were removed from the MFCs and stored immediately in feed solution to prevent drying out. They were then transferred to a fume hood, where they were fixed in a 2.5% glutaraldehyde in sodium cacodylate buffer (SCB) (0.1 M, pH 7.3) for 2 hours at room temperature. Next, they were rinsed in SCB three times for 15 minutes. They were then postfixed in 1% osmium tetroxide in SCB for 1 hour, before being rinsed with distilled water twice for 10 mins.

Samples were then dehydrated using acetone solutions: 50, 70, 90 and 100%. Each solution was changed 3 times over 15 minutes. A 1:1 solution of acetone and hexamethyldisilazane (HMDS) was then poured over each anode and left for 15 mins, before they were left in pure HMDS for 30 mins (the HMDS was changed 3 times during that time). The HMDS was pipetted off the samples, and the remainder allowed to evaporate over 1-2 hours.

Samples were mounted onto SEM stubs, and attached with conductive carbon tape. They were left in a vacuum desiccator overnight. They were then gold-coated using an S150B Sputter Coater (Edwards, UK). The operating pressure was 2×10^{-1} atm, and the coating time was 4 mins.

The samples were then imaged using a JSM-6480LV Scanning Electron Microscope (JEOL, UK) with a 10 kV acceleration voltage.

4.10 – Control of Anode Potential

Four cells were set up and fed the feed solution. All four cells were fed clean feed for up to 3 hours, until their output was stable. A potentiostat (µAutolab III, Metrohm, UK), was then used to control the anode potential of two of those cells. In this case, $E_{electron\ donor}$ was calculated to be +0.172 V (see section 3.3 for method of calculation), so anode potential of +0.4 V was selected in order to create an overpotential of 0.228 V. This overpotential had been used by Stein *et al.* and produced successful results.[13] This cell was also fitted with an Ag/AgCl reference electrode, connected to the potentiostat, as per the literature, to maintain a constant anode potential throughout the experiment.

The micropollutant under investigation was injected after half an hour of anode control, to the first and second cells, and to a third cell under the usual resistor control (set up as in section 4.5). Injection time was also half an hour. A fourth cell acted as a control, and was

fed clean feed throughout. All 4 MFCs were connected to a picologger, and the voltage output of all the devices was recorded. An experimental schematic is shown in Figure 16.

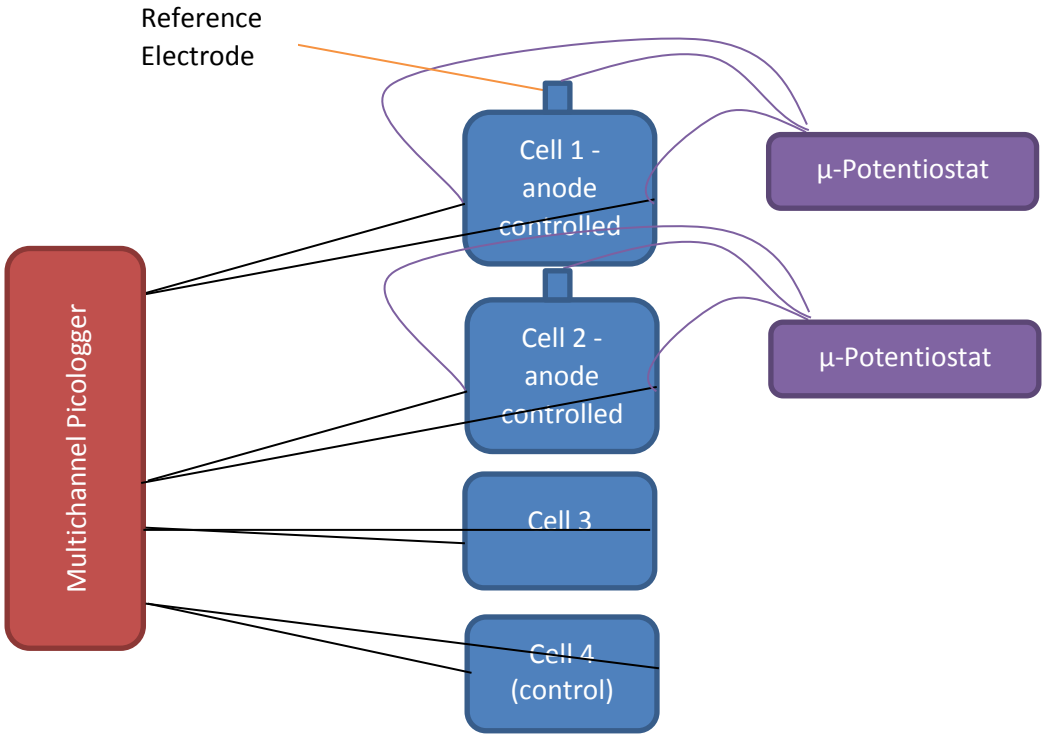

Figure 16 - Schematic Diagram of Anode Control Experiments. All 3 cells were fed with clean feed for half an hour. Anode potential was controlled at +0.4 V for in Cell 1 and Cell 2. Micropollutant was then injected into cells 1, 2 and 3 for half an hour.

5.0 – RESULTS AND DISCUSSION

5.1 – Electrochemical Characterisation

The newer MFC design, as in Figure 12 was characterised using various techniques, as demonstrated in the forthcoming sections. They were inoculated and set up as in Section 4.3, before being fed clean feed solution for 3 days before measurements were taken.

5.1.1 – Polarisation and Power Curves

Polarisation curves are a powerful tool for the characterisation of MFCs. They represent the output voltage of the MFC as a function of the current, and as such represent a way of calculating the optimum external resistance to use in order to gain the maximum power output for the cell, using the methods outlined in Section 2.4.2. [72]

The average power curves in terms of power and current densities are displayed in Figure 17, along with the average polarisation curve. The average power curve in terms of absolute power and current is displayed in Figure 18.

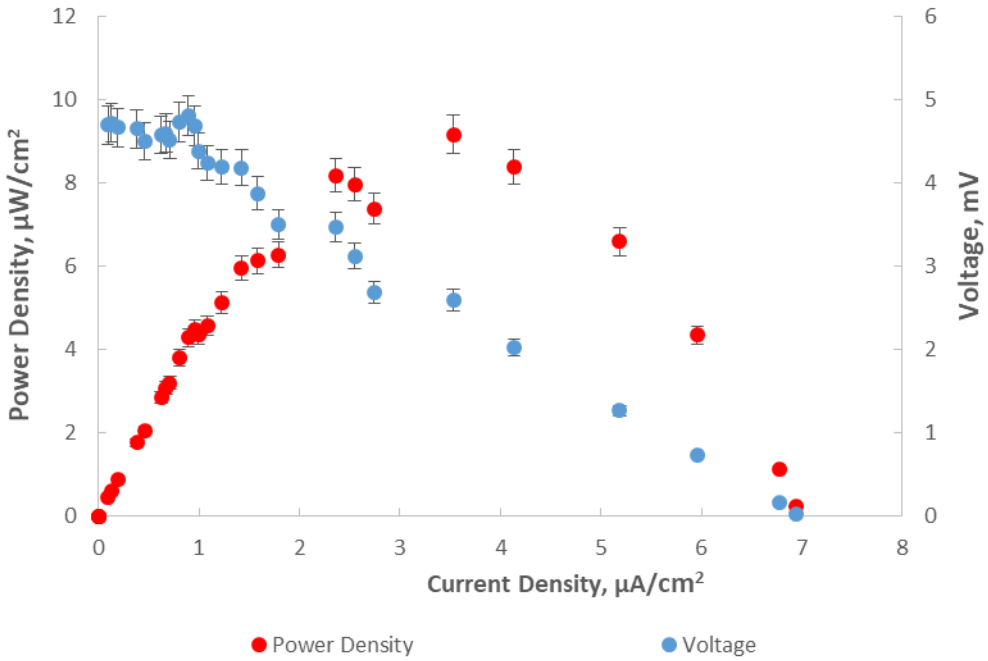

Figure 17 – Average Polarisation and Power Curves for 4 MFCs

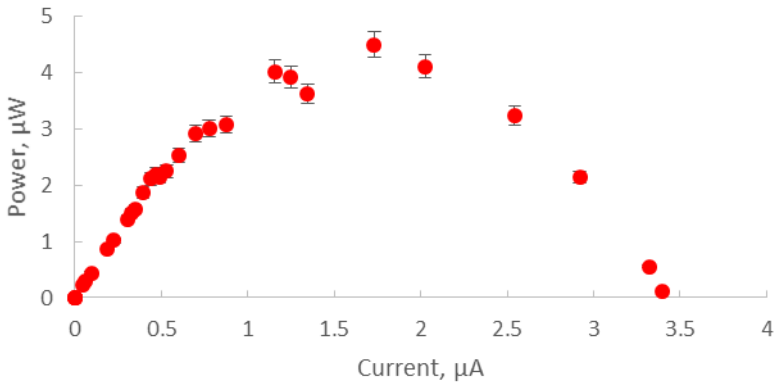

Figure 18 - Average Polarisation Curve for 4 MFCs displayed in terms of absolute current and power

The power curve (power *vs.* current) is of a fairly typical shape for MFCs – bell curves are common. Starting under open circuit conditions, no power is produced (due to P = I × V and V = I × R). [72] Gradually, the power density produced increases with current density until a maximum point at 9.16 µW cm^{-2} (Figure 17). Beyond this point, the power density decreases as a result of increasing ohmic losses due to higher overpotentials, until such a point when power produced reaches zero (short-circuit conditions).

Similarly, the polarisation curve is fairly typical for that of MFCs. The shape usually obtained is linear, and the gradient of that line is equal to the internal resistance of the MFCs ($R_{int} = \frac{-\Delta E}{\Delta I}$). [72] In this case, the internal resistance is calculated to be 326 Ω, which is quite high compared to other values for MFCs (which are usually a factor of 10 smaller).[72] This could be due to poor electrical connections within the MFC – particularly those between the cloth electrodes and metal contacts. It is usually expected that small MFCs will have small internal resistances due to electrodes being in close proximity, minimising the effects of mass transport, so this result proves that that isn't always the case, depending on the construction of the MFC. In future, improving these electrical contacts should be of crucial importance.

Figure 19 shows the output voltage of the MFCs plotted against the resistance applied.

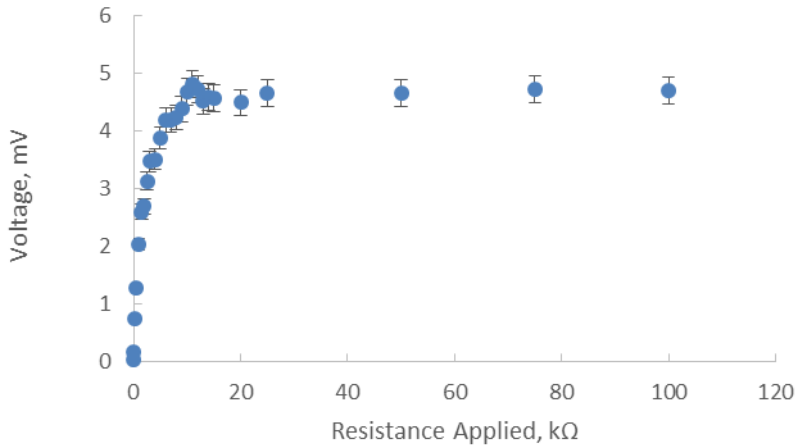

Figure 19 - Average Voltage Output vs. Resistance Applied

The optimum power output was found to be 4.49 µW (Figure 18) and was obtained with a current of 1.7 µA. Using the polarisation curve in Figure 17, we can see that this current was obtained with an output voltage of 2.60 mV. Then, using Figure 19 and Ohm's Law, we can calculate the optimum external resistance to use as 15 kΩ. This value was used to perform all experiments on micropollutant injections after the preliminary investigations.

5.1.2 – Cyclic Voltammetry

Cyclic voltammetry of the anode of the MFCs allows us to interrogate the performance of the electrode surface, and ensure the activity of the biofilms. Cyclic voltammograms were recorded before inoculation with bacteria, and after 2 days of biofilm growth.

Figure 20 shows a typical cyclic voltammogram before inoculation with bacteria. The scans were performed on 4 MFCs, but only a single cell has been shown here for clarity. Results were very consistent across the 4 MFCs measured.

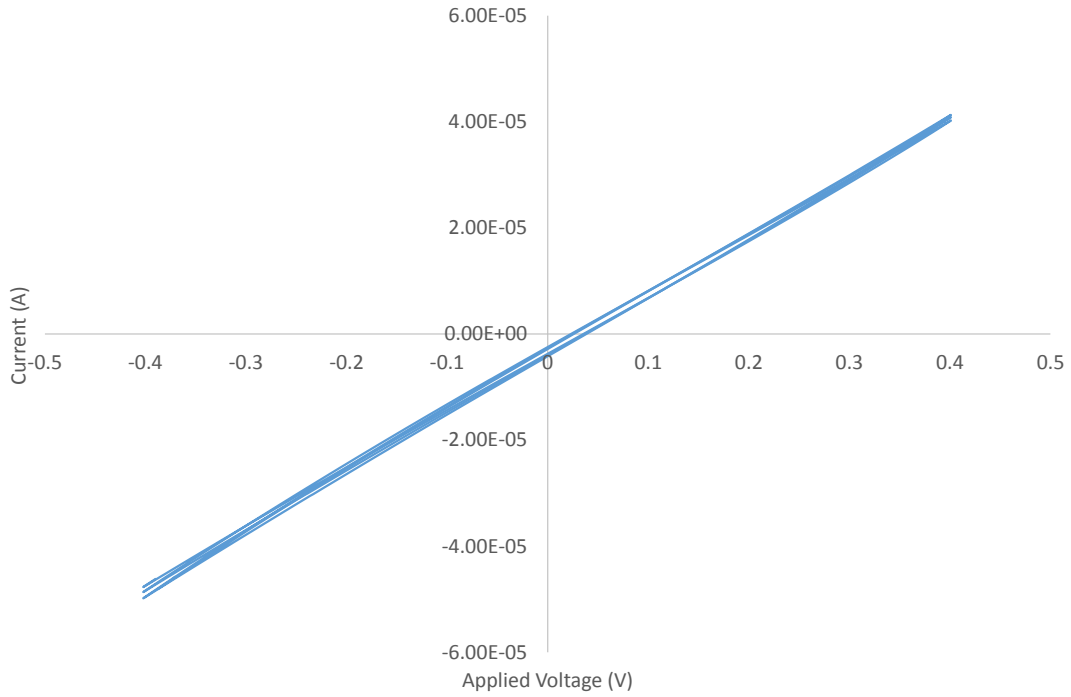

Figure 20 - Cyclic Voltammogram of an MFC before inoculation with bacteria (3 scans)

The difference in current between forward and reverse sweeps is almost negligible, as you would expect for a plain electrode with no inoculum. The carbon source was present in the feed to the cell, but without the biofilm to metabolise it, no reactions take place at the anode. The same trend was observed for all cells.

Figure 21 shows the CV of the same cell after 2 days of inoculation with the bacteria.

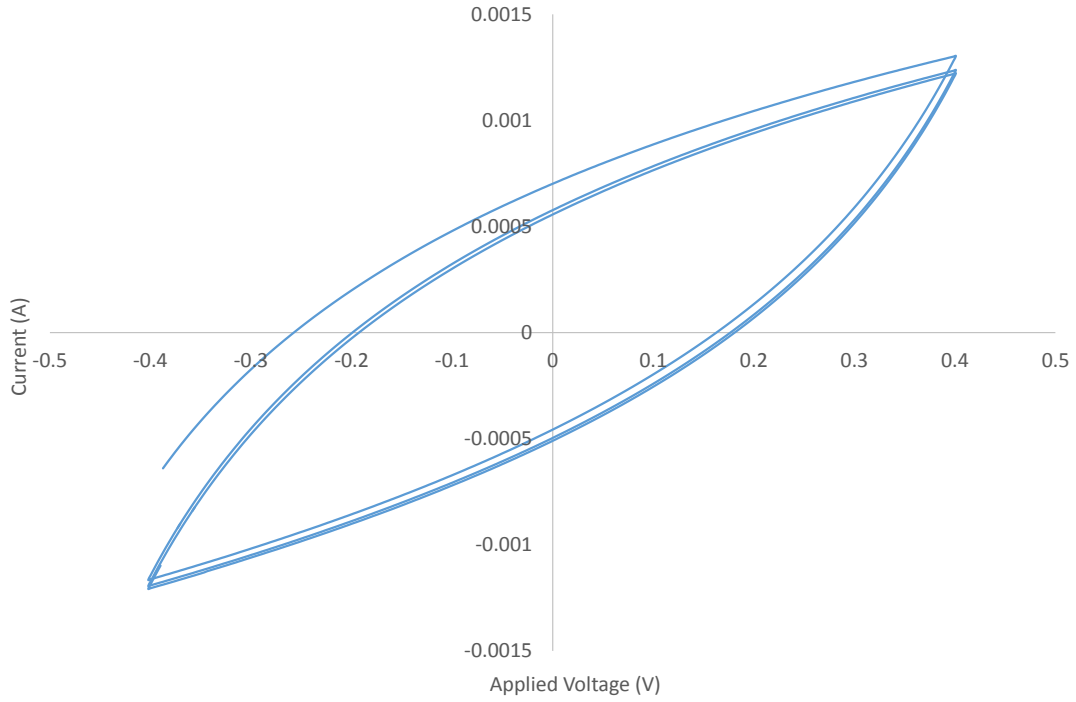

Figure 21 - CV of the Anode of an MFC after 2 days of inoculation (3 scans)

As you can see, there is a large difference in current observed between the forward and reverse scans, and this was true of all 4 cells. This difference was not present in scans of electrodes which had not been inoculated. This difference is caused by the capacitance generated by the growth and attachment of the biofilm to the electrode surface. In most cases, capacitive current can be ignored, [131] as it is usually so small in comparison to Faradaic current that it makes very little contribution. However, the current here seems so large that it may be masking the usual S-shaped signal one would expect from an electrochemically active biofilm. This phenomenon is not unknown in CV of biofilm-coated anodes in MFCs – the high capacitance is attributed to the electrogenic bacteria in the biofilm passing electrons through their complex electron transport pathways at a relatively slower rate compared to transport through normal electrodes. [132]

Again, these results were very similar to those gained on other MFCs. CVs were also performed after 5 days of operation, and yielded similar results and similar capacitances. (Figure 22). The S-shaped curve was never visible in any of the scans, however the increased capacitance does elude to the presence of the attached biofilm at the electrode surface. Additionally, during micropollutant injections, the fact that the current output clearly increases with the presence of the organic micropollutants clearly indicates that the biofilm is active.

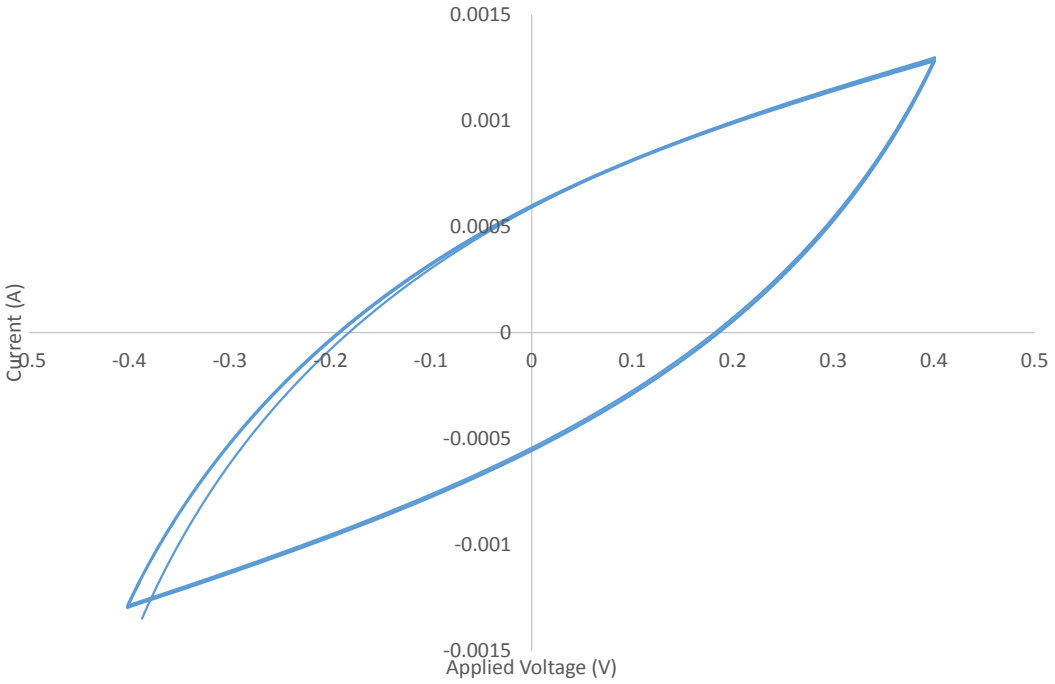

Figure 22 - CV of the Anode of an MFC after 5 days of inoculation (3 scans)

In short, these results show there is a biofilm attached to the anode surface of the MFC. Unfortunately it seems the high capacitance obscures the usual S-shaped curve that would prove the biofilm is active. However, it does confirm its presence, and when combined with other results, we can prove the biofilm is indeed active. Results between different MFCs were very similar, proving the reliability of the inoculation process. There appeared to be little difference between 2 and 5 days of inoculation, which shows 2 days is a sufficient inoculation period to obtain a well-attached biofilm. [132]

The differences in capacitance were calculated according to $C = \frac{I}{s}$, where C is the capacitance, I is the average maximum current in the forwards direction, and s is the scan rate (10 mV s^{-1}). The results are shown in Table 8.

Table 8 - Capacitances of the Device Before and After Inoculation

No. of Days after Inoculation	Capacitance (F)
0 (before inoculation)	0.004
2	0.12
5	0.10

5.1.3 – Electrical Impedance Spectroscopy

EIS allows the investigation the electrochemical properties of the MFCs to gain understanding of their limitations and operation. Several useful quantities can be obtained from this technique, the first of which is internal resistance (R_i).

This can be obtained from a Nyquist plot (Figure 23) which is a plot of real impedance (Z') against imaginary impedance (Z''). Real impedance is an interchangeable term with resistance (R) and imaginary impedance is also known as reactance (X), which is the opposition of a circuit to changes in current or voltage.

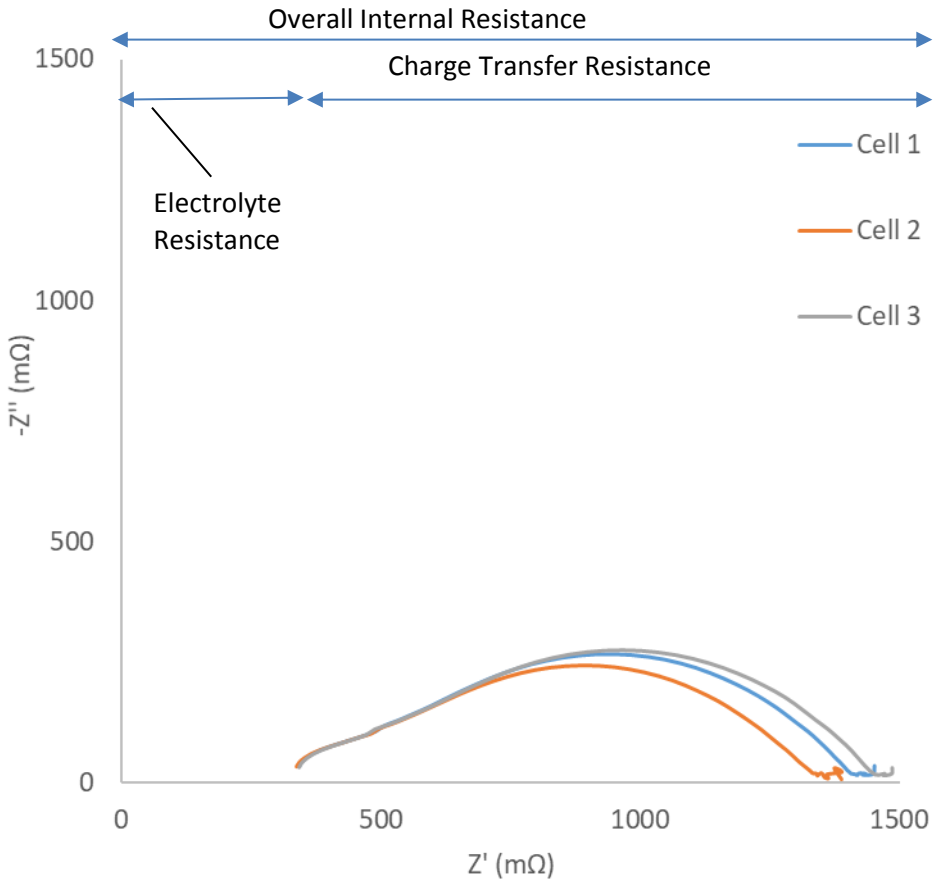

Figure 23 - Nyquist Plot of 3 MFCs

As seen in Figure 23, the value of overall internal resistance can be determined by the point at the end of the characteristic semi-circle shape. This overall internal resistance can be split further into that arising from the electrolyte (before the semi-circle) and that from charge transfer (during the semi-circle). [133] Resistance arising from diffusion is not seen in this

instance – that would manifest itself in a straight line, following on from the semi-circle shape, pointing diagonally upwards.

We can therefore conclude the average internal resistance of the MFC is around 1.450 Ω, of which 0.4 Ω is due to the electrolyte. The remainder is due to the combined processes of charge transfer and diffusion. This value is in keeping with that of other MFCs of a similar size, and made with similar cloth-based electrodes. It is significantly smaller than the internal resistance of MFCs which are larger, highlighting the advantages of miniaturisation in terms of reduced internal resistance and the resulting increase in efficiency.[91, 133]

Using the data from EIS, we can also obtain a Bode Plot (Figure 24). These plots show the variance in the magnitude of impedance (*Z*) and phase angle (θ) with the frequency of the AC current.

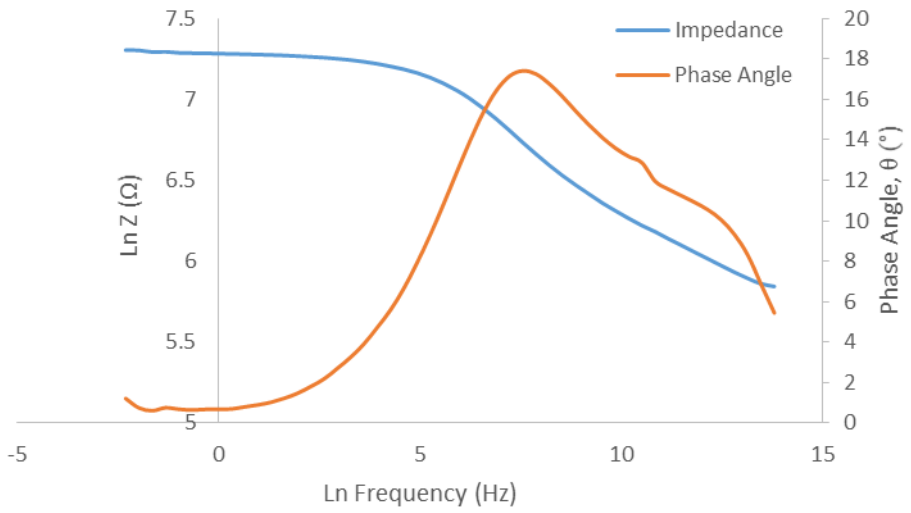

Figure 24 - Average Bode Plot of 3 MFCs

Equation 13 and Equation 14 show the relationships between the quantities obtained from EIS and voltage and current. These allow the calculation of *Z*, the complex ratio of electrical impedance. *j* is used here to represent the imaginary number *i* to avoid confusion with current.

$$Z = Ze^{j\theta} \qquad \text{Equation 13}$$

$$Z = Z' + jZ'' \qquad \text{Equation 14}$$

In this case, the value of *Z* is solved as 0.42 + *j*(-0.0914) Ω.

The fact that a phase angle (θ) is observed shows that the MFC does not behave as an ideal resistor. The fact that it is a negative phase shift, i.e. change in voltage lags behind the change in current through the device shows that it is behaving partially as a capacitor – although a perfect capacitor would have a phase angle of -90°.

5.2 – Testing the Response of MFCs to Organic Biologically active compounds

The major aim of this project was to develop MFCs as biosensors for a range of biologically active micropollutants present in water streams. It was therefore decided to test several of this type of compounds, which, according to literature and reviews by the European Union to choose target molecules, are recognised as important to be monitored. Those chosen were paracetamol and ibuprofen for their intense use[6], naproxen, bisphenol-a, 17β-estradiol and diclofenac[7, 8, 24, 26, 28, 29] for their known effects on vultures and fish, and triclosan for its known antibacterial properties[31, 49].

17β-estradiol has been found in wastewaters in the range of 0.2 ng L^{-1} to 78 ng L^{-1}. [134] Bisphenol-A has been found between 0.088 and 11.8 ng L^{-1}. [135] Triclosan has been found in wastewater at levels between 3.8 and 16.6 µg L^{-1}.[136] Diclofenac has been found in the region of 119-1376 ng L^{-1}.[137]

5.2.1 – Preliminary Experiments

The optimum operating conditions for running the MFCs were elucidated using several different experiments. Conditions such as feed concentration, pH and external resistance were examined. These experiments were carried out initially with the preliminary MFC design (Figure 11) and then some were repeated or investigated only with the final MFC design (Figure 6). Furthermore, some preliminary studies of paracetamol, ibuprofen, naproxen and diclofenac were completed using an early design of MFC (Figure 11).

5.2.1.1 – Experimental Conditions

To be used as biosensors MFCs must be operated under fuel-saturated conditions. This means that, assuming other variables (such as temperature, pH, conductivity, etc.) are also controlled, any change in current output can be attributed to a change in concentration of the analyte. Fuel saturated conditions mean that the concentration of fuel is that which achieves the highest possible power output of the MFCs. [1, 13]

To investigate the concentration at which fuel-saturated conditions are achieved, three previously-enriched MFCs were fed with artificial wastewater containing various

concentrations of potassium acetate as a carbon source at a rate of 2.5 mL min^{-1}. The current output was allowed to stabilise after the concentration was changed. Subsequently, the average current output was taken over a 2-hour period and the results are shown in Figure 25.

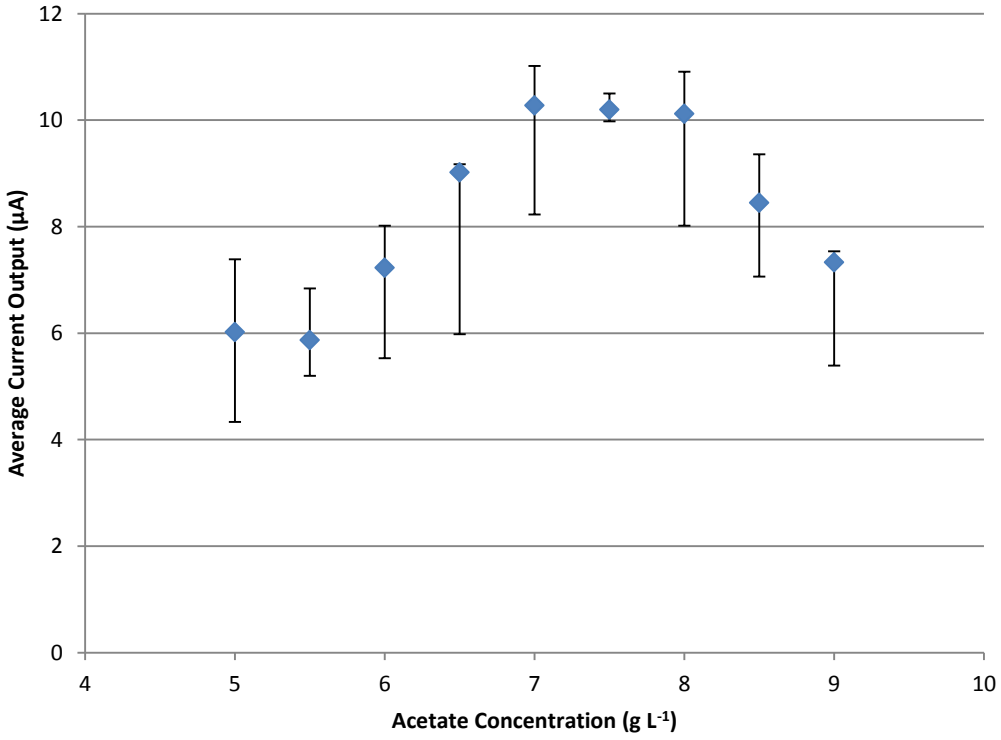

Figure 25 - The Effect of Acetate Concentration on the Average Current Output of 3 MFCs (Preliminary MFC Design)

The error bars represent the error in the average values rather than the range of output currents recorded. It is clear that the optimum acetate concentration was 7 g L^{-1} as it produced the highest current output. However, the error is fairly significant – and the current outputs produced were quite similar, irrespective of the concentration used. Most were within the range of error. However, there is a noticeable drop in performance at the highest concentration, 9 g L^{-1}. It has been demonstrated that when bacteria in a biofilm are fed with high concentrations of food source, they gain large amounts of energy and break away from the biofilm to float freely in solution.[138, 139] This would reduce the current output of the device, as although the bacteria in solution still metabolise the food source, they do not pass the electrons directly to the electrode as often, meaning they are absorbed by other components of the solution. The low performances at concentrations <7 g L^{-1} can be attributed to the fact that the carbon source has become the limiting factor in the rate of

production of electrons (and therefore current). To increase performance any further, more fuel source is required.

The experiment was repeated using the new MFC design, leading to a similar conclusion. 7 g L^{-1} remained the concentration used for experiments. Results are shown in Figure 26.

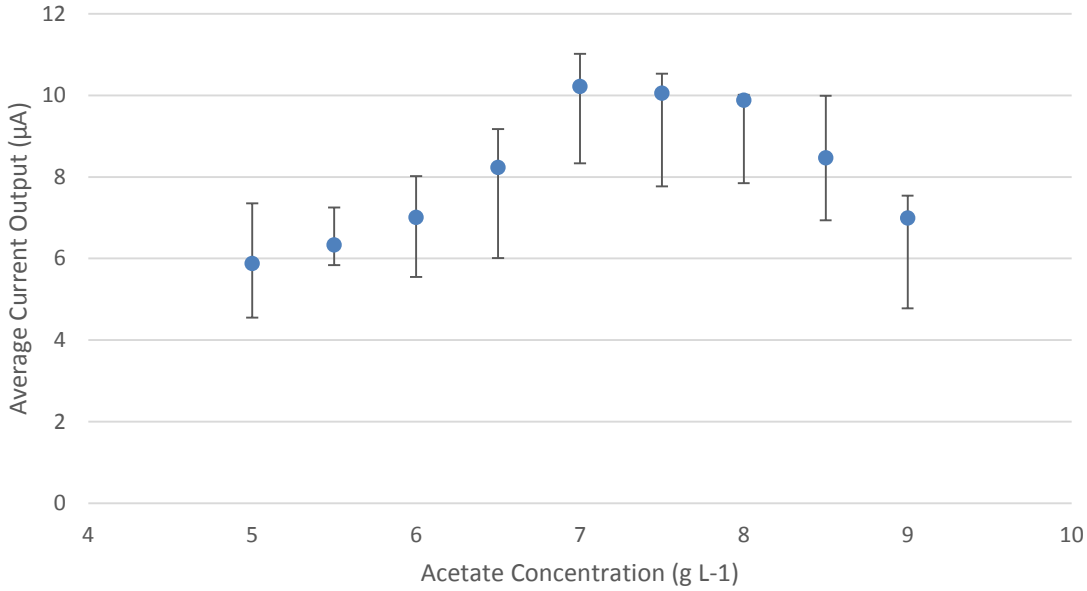

Figure 26 - The Effect of Acetate Concentration on the Average Current Output of 3 MFCs (Final MFC Design)

The effect of flow rate was investigated in a similar way. The flow rate to three MFCs being fed artificial wastewater, containing 7 g L^{-1} potassium acetate, was altered. The output was allowed to stabilise before the average value was taken over a 2-hour period. Results are shown in Figure 27.

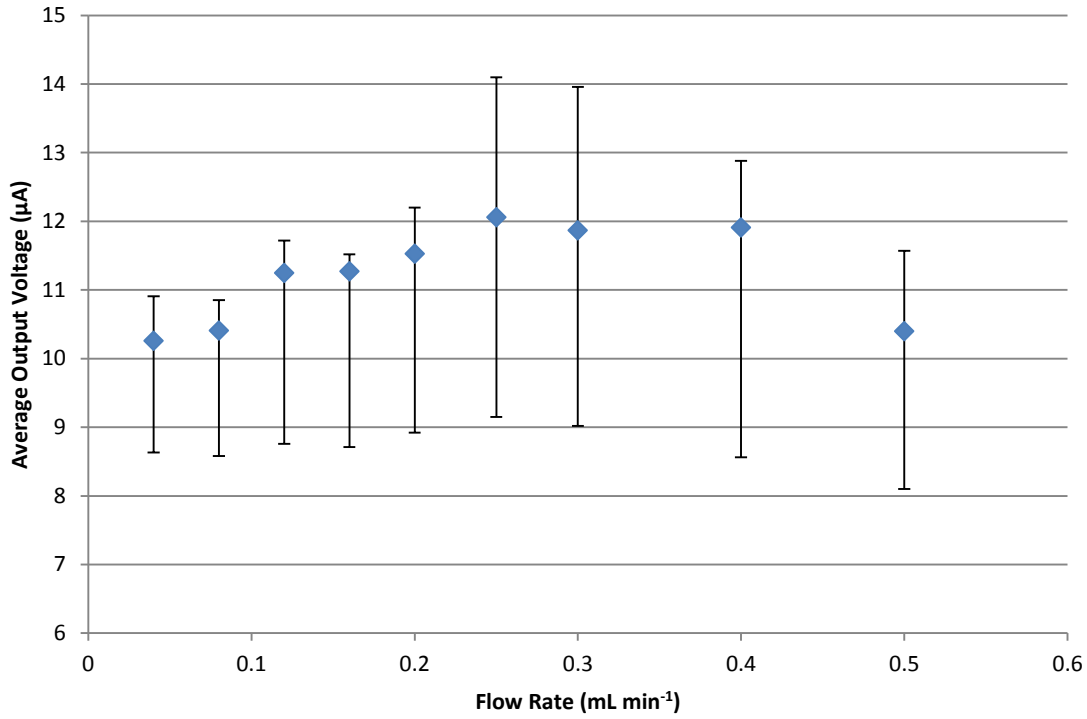

Figure 27 - The Effect of Flow Rate on Voltage Output of 3 MFCs (Preliminary MFC Design)

1.4 mL min^{-1} was chosen as the final flow rate to use in future experiments. Values lower than this produced lower outputs, possibly because the availability of fuel at electrodes decreased with time. Values higher than this had potential to yield better performances, but large error bars mean that this increase was not certain. In theory, however, the more fuel is available at the electrode at a given time, the faster the rate of electron production until a plateau is reached. Considering the increased performance was not certain, and that increasing flow rate means more consumables such as ingredients for the feed solution would be needed to run the fuel cells and perform each experiment, which would in turn cost more, the balance between best performance and material used was considered. The low performance at 5 mL min^{-1} may be attributed to possible shear effects, whereby the biofilm is being torn away from the electrode surface (this could perhaps be investigated by SEM imaging by comparing biofilms grown at different flow rates to see if there is any visible difference in biofilm coverage or structure) or low residence times in the device meaning not all of the fuel could be oxidised.[140]

The flow rate selected was not changed for experiments using different reactor designs in order to make any data obtained using different designs comparable. Furthermore, altering the flow rate would have been difficult, since the pump used was already at its minimum speed setting. If the optimum for the newer cells was lower (which is likely, as they are

smaller) the only way to reduce it would have been to reduce the internal diameter of the tubing used, which would have made it difficult to connect to the MFCs inlets.

At this flow rate, hydraulic retention time (τ) of the MFCs was calculated as in Equation (15).

$$\tau = \frac{Volume\ of\ reactor}{Influent\ Flow\ Rate} \quad (15)$$

The volume of the reactor was 6.4 x 10^{-8} m^3 and the influent flow rate was 8.4 x 10^{-5} m^3 hr^{-1}. This gives a value of 7.619 x10^{-4} hours, or **2.7 seconds**. This is a relatively short retention time, but long enough for oxidation to occur on a kinetic basis. [141]

pH is another variable that can affect the output of MFCs. It has been shown that pH gradients can limit the growth of biofilms, as well as alter electrochemical pathways in the MFC.[142] Therefore it was decided to attempt to use a pH-buffer in the feed solution to ensure that the feed solution remained a similar pH throughout all experiments to eliminate this variable. The buffer used was a 0.1 M, pH 7.5 phosphate buffer (11.93 g L^{-1} Na_2HPO_4 and 2.205 g L^{-1} $NaH_2PO_4.H_2O$). First, an experiment was carried out with 3 MFCs, to determine whether the presence of the buffer had any detrimental effect on the performance of the MFCs. Each MFC was fed clean feed for 3 hours and then clean feed containing a pH buffer for 3 hours. The current output of the MFCs was monitored and averaged over the final 2 hours of each of the feeding periods. The results are shown in Figure 28.

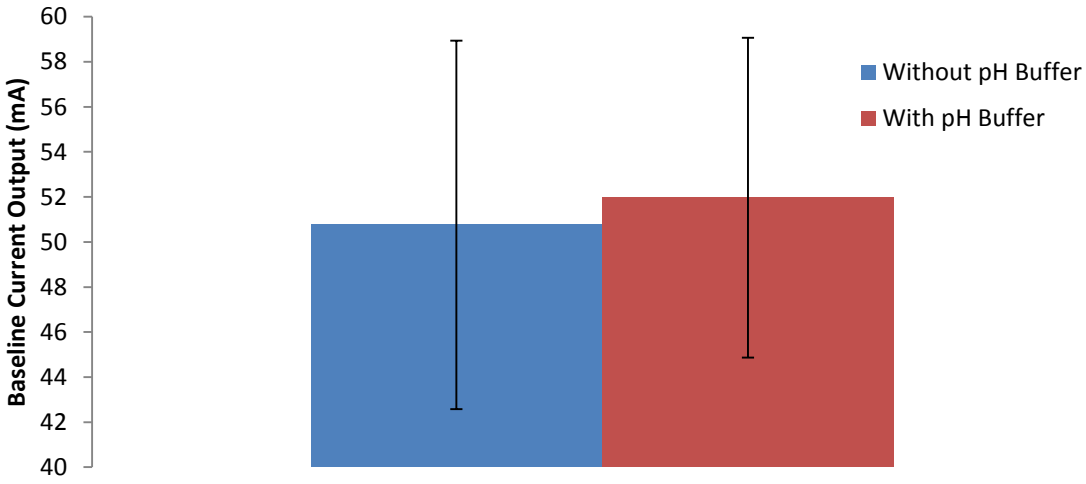

Figure 28 - Effect of Using a pH Buffer on the Current Output of 3 MFCs (Final MFC Design)

The results from using both mediums are very similar, the difference between them is well within the margin of error. Therefore, it was decided to use a pH-buffered medium for future experiments. However, preliminary experiments (in Section 5.1.2) did not use a pH-buffered

medium. The fairly large error bars cover a range of about 20 mA which is a result of a wide variation in current outputs of different cells. This is an effect that was noted throughout the course of many experiments performed, and is addressed in later chapters, with the introduction of control of anodic potential.

It is also known that adding compounds to a solution can alter its pH. To investigate if this would still be the case using a pH-buffered medium, four of the biologically active compounds to be investigated were added to pH-buffered medium at varying concentrations, and the pH was measured. Results are shown in Figure 29.

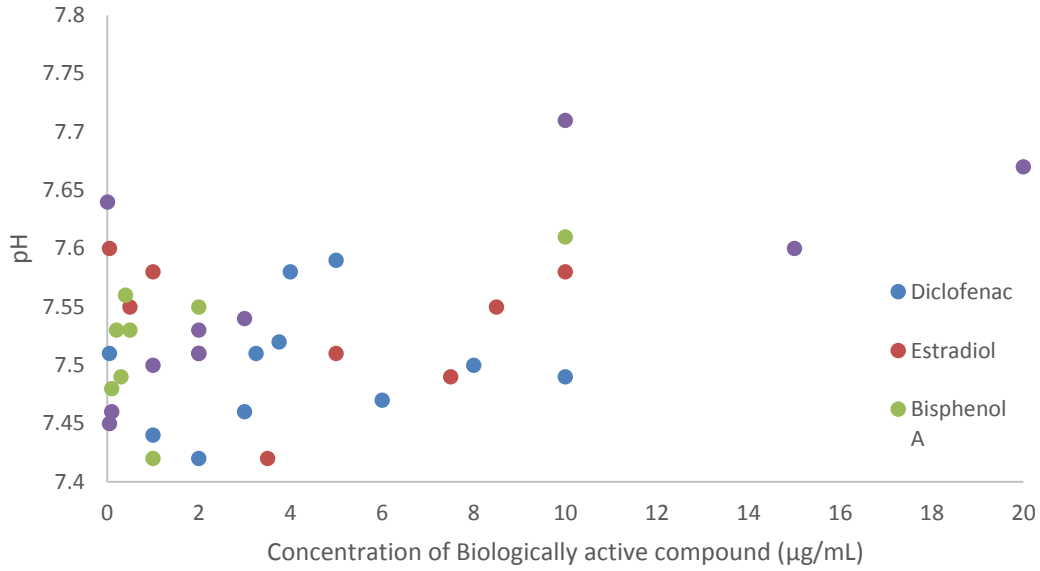

Figure 29 - Effect of the Presence of Biologically active compound Compounds on the pH of Feed Solution (Final MFC Design)

The results show no obvious pattern in the pH of the solutions with increasing concentrations of biologically active compound. Furthermore, the pH ranges are very small, all between 7.4 and 7.7. This indicates that the pH-buffered solution is performing acceptably and negating any effect of adding the biologically active compound molecules. Diclofenac has been shown to be acidic, with a pK_a around 4, which is a reasonably strong acid. Therefore it can decrease the pH of a solution. [143] Phenols, such as estradiol and BPA have been shown to have pK_a values of around 10, meaning they are most likely alkaline in solution, given that the protons do not dissociate often. Triclosan has a pK_a of 7.9, however, which places it roughly in the middle, perhaps very slightly alkaline.

These two experiments combined demonstrate that the use of a pH-buffered medium is suitable for use in these experiments, as the concentrations of pharmaceutical used are well

controlled by the buffer medium, and the presence of this pH buffer is not detrimental to the performance of the MFCs.

Conductivity of the medium can also affect the electrochemical characteristics of a system, and thus the current output of a device. Adding ions to a solution increases its conductivity. In theory, this should be a linear relationship, however it depends upon the mobility of charge carrying ions in solution. The mobility of such ions is decreased by increasing concentrations, so this linearity only holds true up to a point. Therefore, it was decided to conduct an experiment to see how the different pharmaceuticals under investigation affected the conductivity of the feed solution. Results are shown in Figure 30.

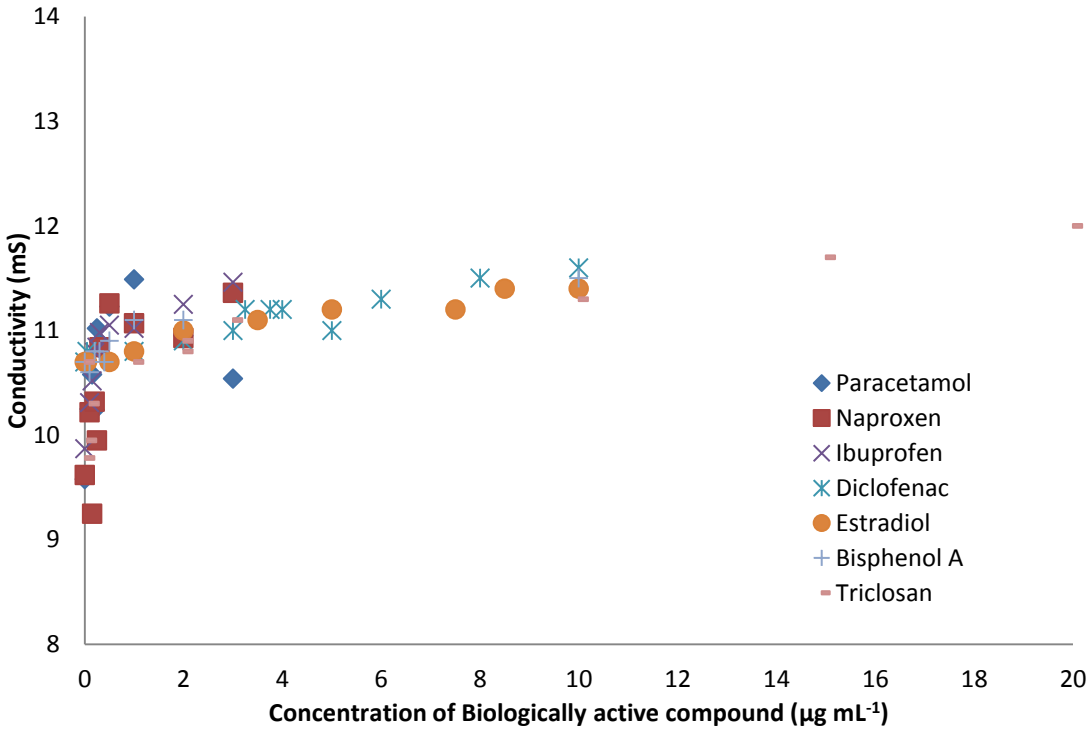

Figure 30 - Effect of the Biologically active compounds on the Conductivity of the Feed Solution (new cell design)

For all biologically active compounds, concentrations between 0.15-1 µg mL^{-1} increased the conductivity of the feed solution quite rapidly, but after these concentrations, the increase was much less marked. In any case, the increase in conductivity was relatively small. The conductivity of the feed solution was 9.62 mS with no biologically active compound present, and the maximum conductivity observed was 12.00 mS at 20 µg mL^{-1} triclosan. It was therefore concluded that the biologically active compounds used within the normal concentration range (up to 10 µg mL^{-1}) had no significant effect on conductivity, and that it was therefore acceptable to ignore this variable. [144]

Temperature has been shown to affect the metabolisms of many organisms, and the kinetics of several chemical processes. As such, temperature is a variable known to affect the performance of MFCs. To get the best performance from the MFC, the optimum operational temperature needs to be found, to give the highest signal output.[81] The results from optimisation of the operation temperature are shown in Figure 31.

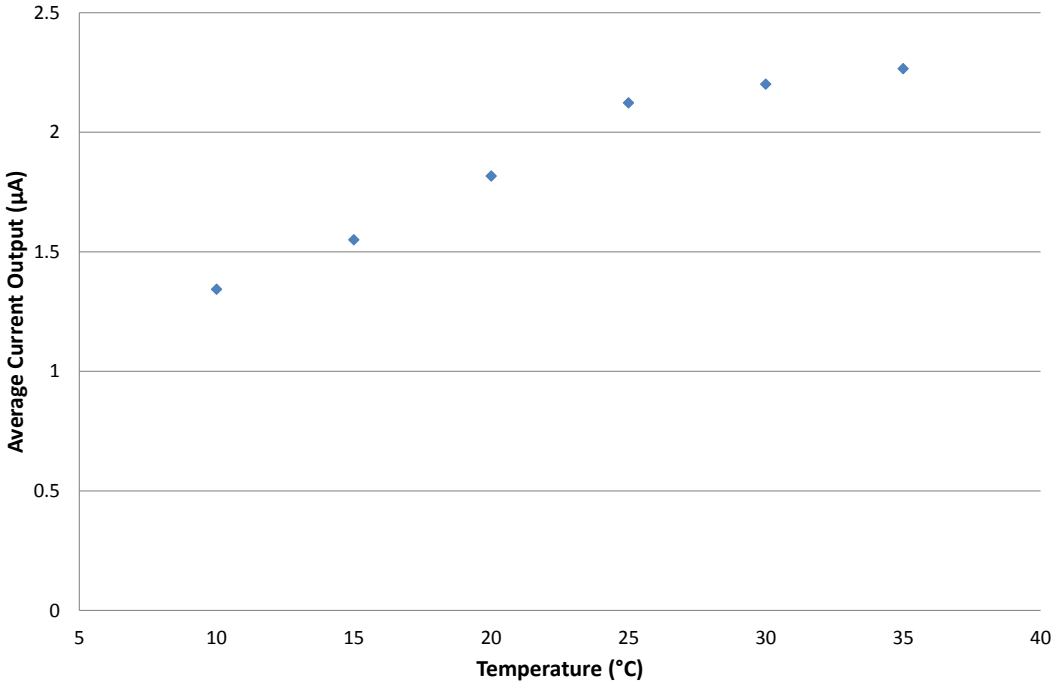

Figure 31 - Temperature's Effect on MFC Performance (Preliminary MFC Design)

The general trend is that fuel cell performance increases with temperature in the range trialled. Higher temperatures were not investigated due to limitations in the range of the incubator. Although the average output current of the MFCs did increase throughout the range investigated, it was deemed that 30 and 35 °C did not represent a significant performance increase upon 25 °C, so this was selected as the operating temperature for future experiments.

This is somewhat surprising, as most bacteria and enzymes operate at much higher performances closer to temperatures which emulate conditions inside the human body, such as 37 °C. As the bacteria which form the biofilm are of unknown species, it is difficult to attribute this to any particular cause. It may be that the species contained within the biofilm have different optimal conditions than typical bacteria, or that the temperature is having another kinetic effect or similar elsewhere in the MFC. Without further experiments to determine the nature of the biofilm, it is difficult to attribute this further.

5.2.1.2 – Detection of Biologically active compounds using the Preliminary MFC Design

A toxic event was mimicked by feeding the MFCs for 3 minutes with the fuel containing the target biologically active compound. The output voltage was continually monitored by the pico logger. After the toxic event, the MFCs were fed with the fuel and no biologically active compound for at least 1 hour to allow recovering before a test was performed.

The output voltage was recorded 30 seconds before and 30 seconds after the toxic event and subtracted from each other to show the response of the fuel cells to different concentrations of biologically active compound. Three repeats were performed for each biologically active compound. Results from the injection of paracetamol are shown in Figure 32.

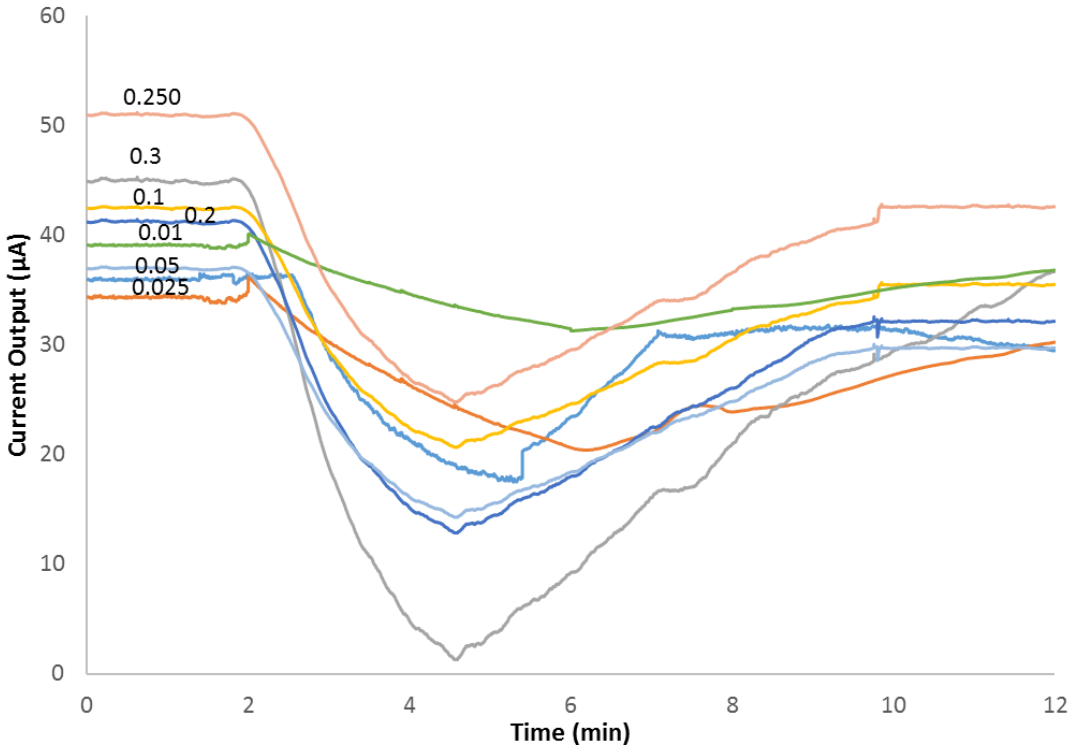

Figure 32 – Preliminary Results from the Investigation of Paracetamol. The numbers in the graph represent the concentration (µg mL^{-1})

All the concentrations of paracetamol tested caused a negative response. This implies that paracetamol inhibits the production of electrons by the bacteria in the biofilm, by either interfering with the metabolisation of the fuel source (acetate) or perhaps by killing some

of the bacteria. Without further investigation, it is difficult to determine which is the case, but paracetamol has been shown to have adverse effects on biofilms, resulting from the death of some bacteria contained within them.[145] The shapes of the responses were all similar, with the exception of the lowest concentration tested, 0.01 µg mL^{-1}. This could be because this low concentration has less of an acute toxic effect than the higher concentrations used.

Results when using ibuprofen as the biologically active compound are shown in Figure 33. The response curves from this biologically active compound are a very different shape to those from paracetamol, but they seem to all be of a similar shape. This could be an effect of the type of toxicity the biologically active compound has on the bacteria, and its method of interaction with the bacteria.

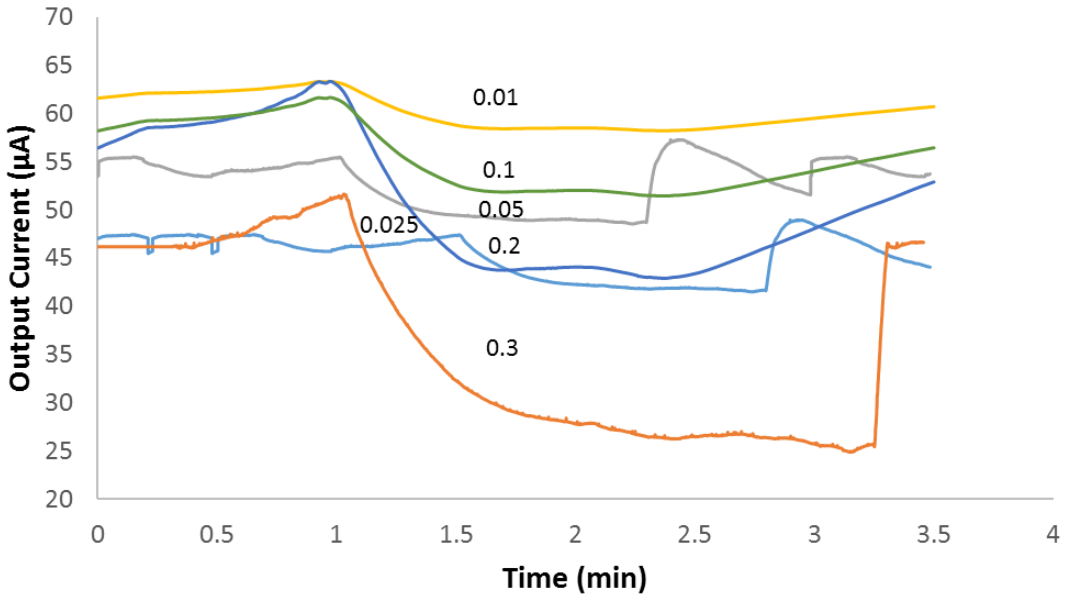

Figure 33 – Preliminary Results from the Investigation of Ibuprofen. The numbers in the graph represent the concentration (µg mL^{-1})

All concentrations measured caused a response which decreased the current output of the cells, again implying that the presence of the biologically active compound inhibits the metabolism of the bacteria in the biofilm, or kills some of the bacteria in the biofilm. The lowest concentration measured, 0.01 µg mL^{-1} was difficult to quantify, as the change in voltage was very small. There is a small drop in output, of around 5 µA once the biologically active compound is injected, and the output does increase after the micropollutant is removed, but this is difficult to see and attribute to specifically the biologically active

compound as fluctuations of this magnitude are common in the baseline current. The results for 0.025 µg mL^{-1} and 0.05 µg mL^{-1} showed an initial very strong recovery after injection (greater than the initial drop after injection) but then declined subsequently. It is unclear why this should be. The negative response of 0.3 µg mL^{-1} ibuprofen was stronger in magnitude than the others, and had a longer recovery time, which is perhaps not surprising, as it was the highest concentration investigated and may have had the strongest effect on the bacteria in the biofilm.

Preliminary results from diclofenac are shown in Figure 34. The responses from this biologically active compound are perhaps the least clear as the shapes of the curves are very different depending on the concentration used. This could be due to different toxicity effects with different dosages, although this is difficult to confirm.

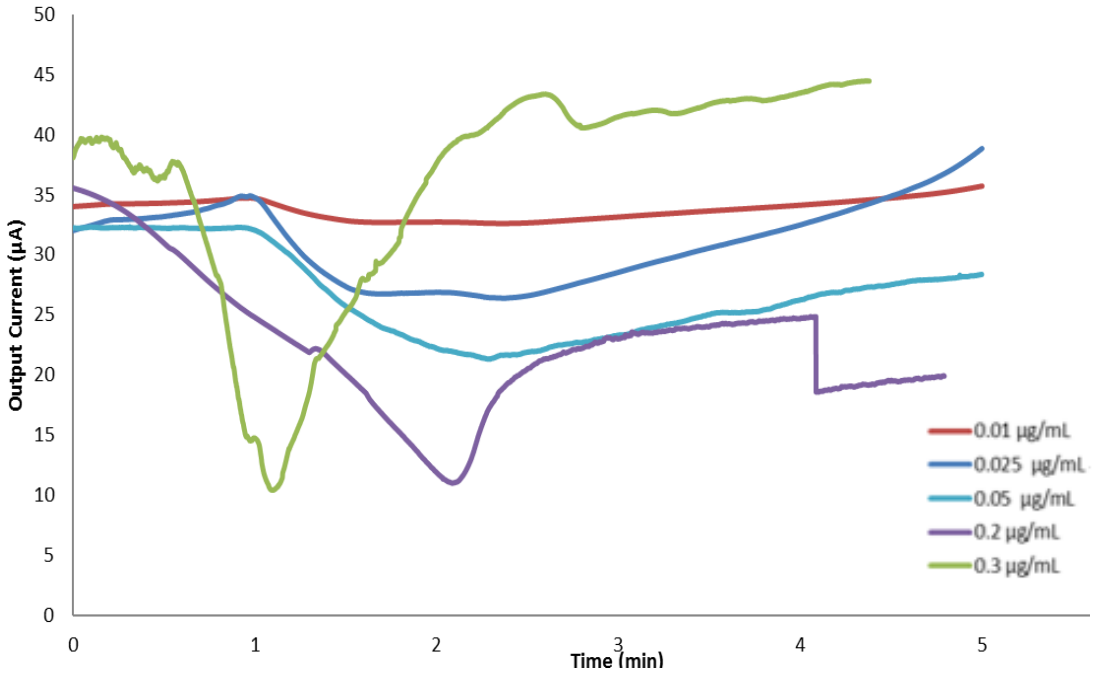

Figure 34 – Preliminary Results from the Investigation of Diclofenac

Again, the lowest concentration measured (0.01 µg mL^{-1}) caused a visible negative response which then recovered, but the magnitude of it was small enough that this can't easily be attributed to the biologically active compound alone. All other concentrations caused a negative response. The highest concentration (0.3 µg mL^{-1}) caused the quickest and sharpest fall in output of the cells, but also exhibited the quickest recovery. Other concentrations showed a negative response which recovered more slowly. It is not clear why there is a difference in recovery time – it would be more typical to have a slower recovery time for a higher concentration of biologically active compound.

Preliminary results from the final biologically active compound investigated, naproxen, are shown in Figure 35. All concentrations tested caused a negative response in the fuel cells.

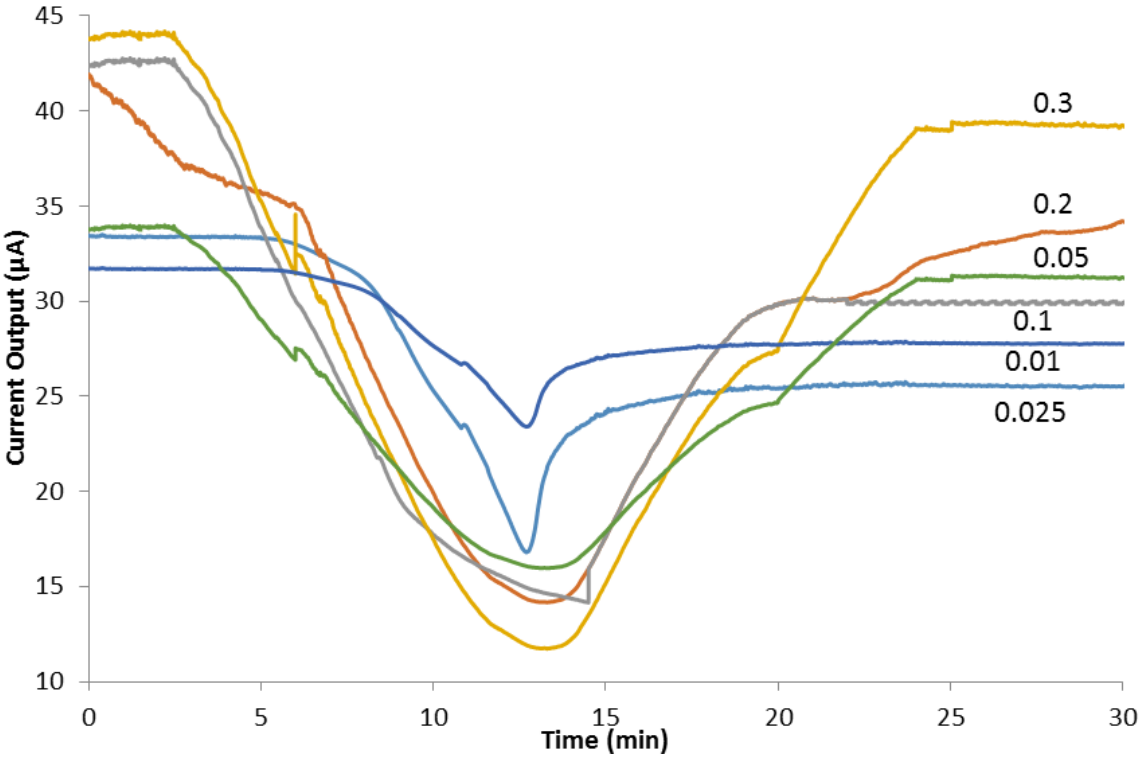

Figure 35 – Preliminary Results from the Investigation of Naproxen. The numbers in the graph represent the concentration (µg mL^{-1})

The curves of all responses were relatively similar, with the exception of the two lowest concentrations tested (0.010 and 0.025 µg mL^{-1}). These exhibited a sharper point at the trough of the curves. This perhaps shows a slower response to a low dose of biologically active compound, and a faster recovery. This is unsurprising considering lower doses are known to be less toxic. The higher doses seemed to cause quicker and deeper drops, with slower recoveries.

With all biologically active compounds tested, over all concentrations, negative drops were observed, followed by recovery to a similar current output to before injection of the biologically active compound – though sometimes the recovery was partial. This implies that the biologically active compounds either kill some of the bacteria, or inhibit their metabolism. Then the biofilm recovers and current output is restored.

However, the baseline currents of all fuel cells were not always similar. For example, the baseline currents before the experiment in the case of the paracetamol experiment (Figure

32) were over range of 20 µA. This means that in terms of pure magnitude, the change in current output before and after biologically active compound injection was not always numerically larger for greater concentrations of biologically active compounds. This makes results from different fuel cells difficult to compare. However, the change in current as a proportion of the current baseline was found to be a more reliable measure, allowing us to compare fuel cells with different baseline currents. This term is known as signal ratio, and is calculated as in Equation (15). I_0 is the baseline current before biologically active compound injection, and I_t is the current at the time being investigated after biologically active compound injection.

$$\text{Signal Ratio} = \frac{\sigma I_0 - I_t}{\sigma I_0} \times 100\% \qquad (15)$$

It can be expressed as a simple ratio or a fraction. The responses for each biologically active compound were plotted in terms of signal ratio against concentration, and are shown in Figure 36.

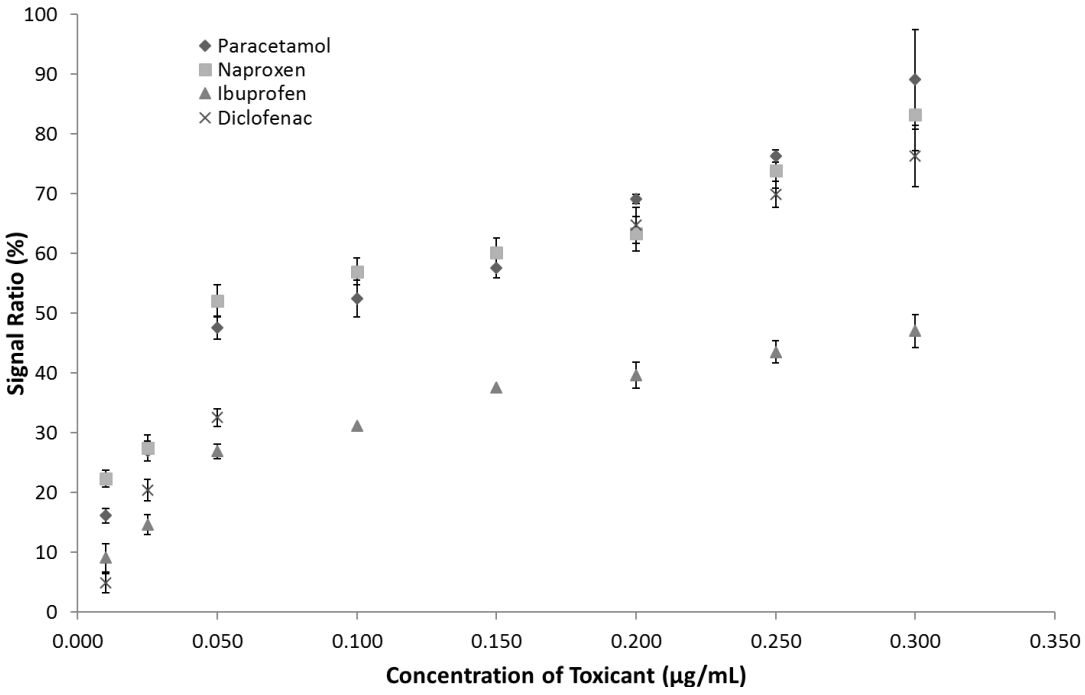

Figure 36 - Response of All Biologically active compounds in Terms of Signal Ratio

As this graph clearly shows, increasing the concentration of each of the biologically active compound increases the response of the MFC in terms of current output, when compared in terms of signal ratio.

The sensitivities of the MFCs towards each of the biologically active compounds were calculated as per the methodology section and are shown in Table 9.

Table 9 - Sensitivities of Preliminary Biologically active compounds Using Old MFC Design

Compound	*Sensitivity* ($\mu A\ M^{-1}\ A\ cm^{-2}$)
Paracetamol	**0.805**
Ibuprofen	**0.413**
Naproxen	**0.815**
Diclofenac	**0.822**

The MFCs were most sensitive to the more toxic compounds, naproxen and diclofenac. They also exhibited high sensitivity to paracetamol, which is somewhat surprising considering its relative toxicity. They were much less sensitive to ibuprofen, which perhaps explains why the curves for ibuprofen were a different shape – much broader. This could be a result of the much lower toxicity that ibuprofen has compared to diclofenac and naproxen.

This work acted as proof of concept that the sensors could detect biologically active organic biologically active compounds, in a similar range of concentrations to those found in wastewater. However, the original data from some of these experiments was lost, owing to equipment failure. The results could have been repeated, however, these old MFCs were very difficult to operate as they were prone to leaks and poor electrical contacts, making replicating these experiments difficult.

The MFC design for future experiments was changed a smaller cell (4 mm^3) with both electrodes comprising of carbon cloth, as described in the methodology. This greatly reduced leaking and improved electrical contact with the electrode. Injection time also needed to be increased to 30 mins to gain clear signals. Paracetamol and ibuprofen were tried with new geometry, but did not produce a discernible response.

This perhaps indicated that the new MFCs are less apt to detecting some biologically active compounds, but that the results they gained were more reliable and repeatable. All future

experiments were performed in line with the protocol detailed in the methodology, including the use of pH-buffered solutions, higher external resistances etc.

Furthermore, signal ratio was altered to reflect the fact that some biologically active compounds produced a negative response and some produced a positive response. The new formula used is shown in Equation (16).

$$\text{Signal Ratio} = \frac{I_t - \sigma I_0}{\sigma I_0} \qquad \text{Equation (16)}$$

This equation better accounts for the different responses of different biologically active compounds. A negative response (*i.e.* decreased metabolisation) is represented by a negative signal ratio, and a positive response (*i.e.* increased metabolisation) is represented by a positive signal ratio value. Future results will be presented in this manner.

5.2.2 – Bisphenol-A

Results from injection of bisphenol-a as the biologically active compound are shown in Figure 37. The results demonstrate a clear positive linear trend between 0.05 µg mL^{-1} and 2 µg mL^{-1}. Increasing the concentration of biologically active compound any further did not cause a large increase in the size of the signal, relative to the baseline, indicating a plateau had been reached.

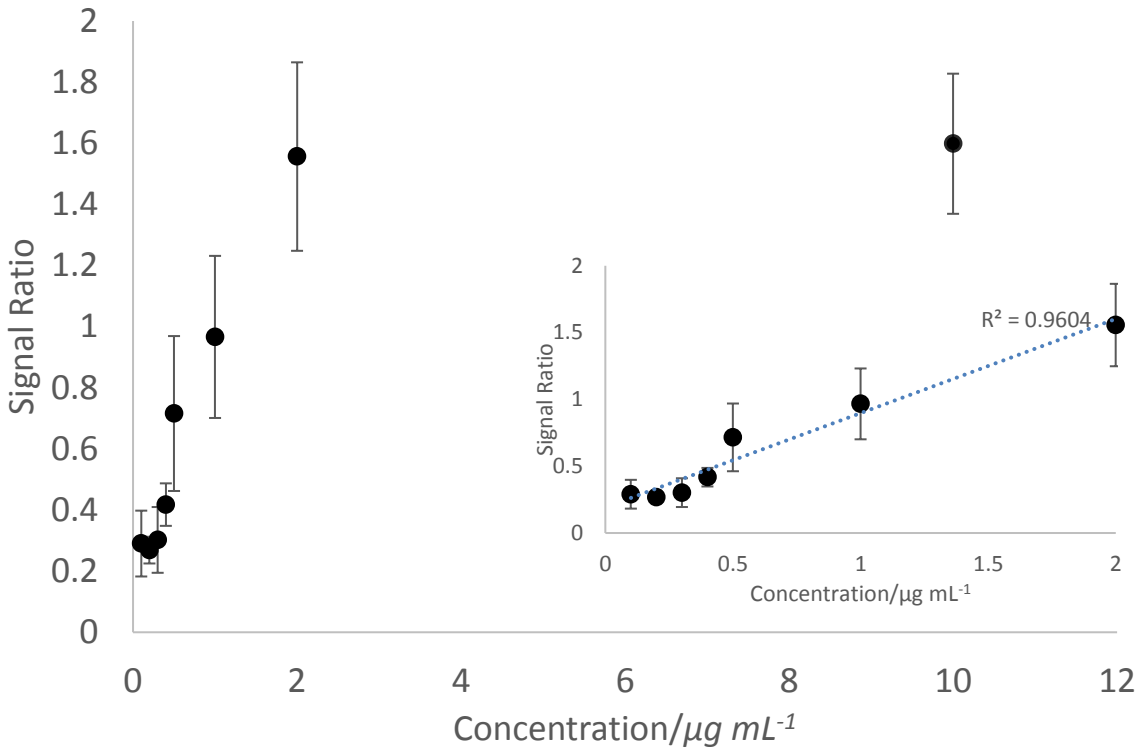

Figure 37 - Effect on the Signal Ratio of 3 MFCs with Various Concentrations of Bisphenol-A. Injection time 30 mins. Insert shows the range between 0.1 and 2 µg mL^{-1}. The error bars represent 1 s.d. There were 3 replicates.

The minimum detectable concentration was found to be 0.1 µg mL^{-1}. The sensitivity was calculated in the linear region and found to be 1.001 nA $µg^{-1}$ cm^{-3}. At each concentration tested, the response to the biologically active compound was positive. This indicates that in this concentration range, it does not have a toxic effect on the bacteria within the biofilm, and is probably metabolised, and as such generates an extra electron flow as it is broken down.[18] There is also some evidence to suggest that BPA is responsible for an increase in protein production in biofilms of some species of bacteria. They are shown to induce the production of proteins which are extruded from the cell, some of which remain in the membranes and act as pumps to remove biologically active compounds from the cell more

effectively. This could also be responsible for some of the increase, as more metabolic pathways are activated, to produce the protein, and biologically active compounds are removed more effectively from the cell.[135] The species involved in the authors' study were *E. Coli* and *P. Aeruginosa*, both of which are commonly found in wastewater and biosolids, so are likely present in the biofilms grown for this investigation.

Samples were collected at the inlet and outlet of an MFC during injection of different concentrations of the biologically active compound. These were analysed using LCMS. Results from LCMS analysis of bisphenol-a are shown in Figure 38. The data show that in each case, almost all of the biologically active compound is removed from the sample during the course of its passage through the MFC. Control experiments with a blank (where the MFC had no inoculation with bacteria) show that a large part (an average of 54.2%) of that decrease could be due to absorption processes occurring at the carbon cloth electrodes in the MFC. Furthermore, bisphenol-a is a well-known plastic additive and has been shown to be absorbed by several plastics previously.[146] However, samples passing through active MFCs achieved a removal of 94.9%, indicating around 40% of the material is removed by metabolisation. This decrease in concentration, combined with the positive responses of the MFCs to BPA provide strong evidence that within this concentration range, the biologically active compound is metabolised by the bacteria in the MFC, producing a greater current output than the baseline current.

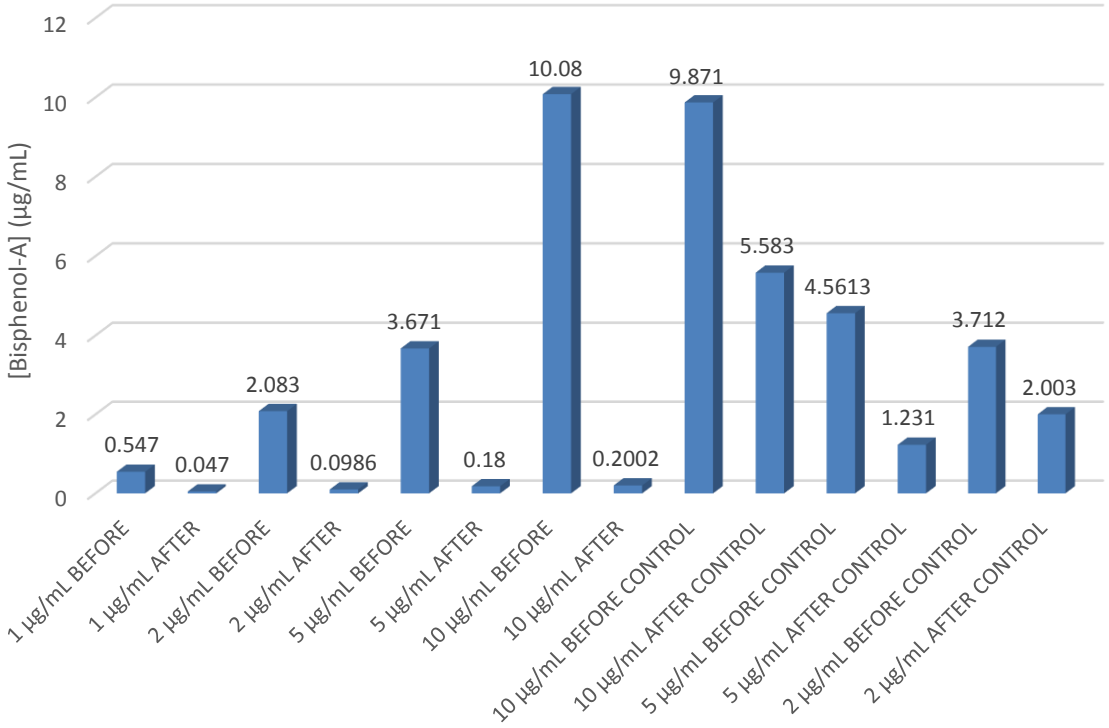

Figure 38 - LCMS data (including blank control experiments with no biofilm) showing the change in concentration from inlet to outlet of bisphenol-a during a toxic event

5.2.3 – Estradiol

Results from injection of 17β-estradiol as the biologically active compound are shown in Figure 39. Again, a positive correlation between increasing concentration of estradiol and the signal ratio is observed, and is best fitted to a 2nd order polynomia. The minimum detectable concentration in this instance was 0.5 µg mL^{-1}. The sensitivity was calculated to be 18.1 nA $µg^{-1}$ cm^{-3} making the MFC-based sensors much more sensitive to 17β-estradiol than bisphenol-a, by an order of magnitude.

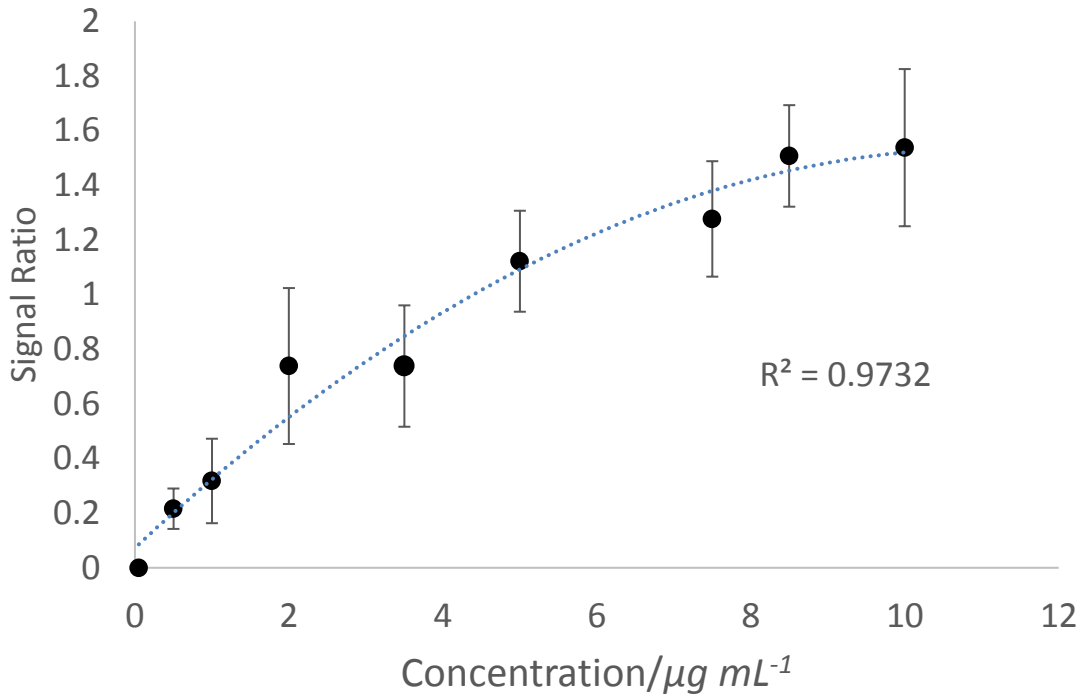

Figure 39 - Effect on the Signal Ratio of 3 MFCs with Various Concentrations of 17β-estradiol. Injection time 30 mins. The error bars represent 1 s.d. There were 3 replicates.

Again, it is likely that within this concentration range, 17β-estradiol is metabolised by the bacteria within the MFC, leading to the positive response, in a similar fashion to bisphenol-a. It is not surprising that its behaviour is similar, since it has been shown to have a similar endocrine-disrupting effect *in vivo* to BPA.[24, 25, 28]

Figure 40 shows the LCMS data for 17β-estradiol, and, the concentration of the biologically active compound decreases on passing through the MFC and is often decreased by over half.

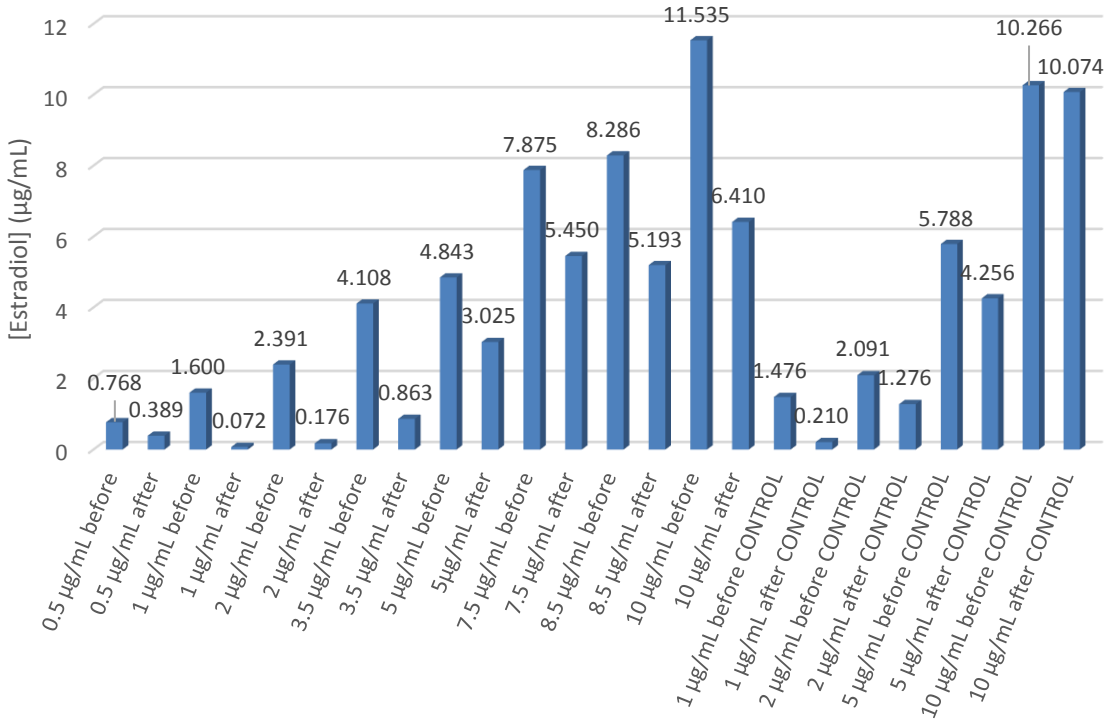

Figure 40 - LCMS data for 17β-estradiol

The control samples showed that the MFC with no biofilm removed an average of 38.3% of the estradiol. Whereas samples passing through an active MFC containing a biofilm showed an average of 58.3% estradiol. This demonstrates that about 20% of the estradiol is removed by metabolisation, about 40% by absorption and other processes in the MFC, and the rest remains in solution.

There appears to be no trend in the percentage removed by metabolisation alone (when that removed by adsorption processes etc. in the blank is subtracted from the results with an active biofilm). This could indicate that the two processes are “in competition” for the same resources and therefore happen at different rates depending on the concentration of the estradiol present. This effect has been demonstrated in MFC-based systems to remove phenol from wastewaters, in which the rate of absorption varied depending upon the initial concentration of phenol. Broadly speaking, increasing concentration led to increased adsorption, but increased adsorption was not demonstrated at concentrations where oxidation of the fuel was kinetically fast enough that none was available for adsorption. [147]

These results highlight the similar behaviour of 17β-estradiol to bisphenol-a. In the cases of these two biologically active compounds, where positive responses were observed in the voltage output of the MFCs, and LCMS data show that removal of the biologically active

compound by the MFCs in greater amounts, compared against a control with no biofilm, is possible using MFC technology. Treatment of wastewater using MFCs has been observed previously in terms of a reduction in overall COD, and a removal of well-documented carbon sources such as sugars and acetate.[148-150] Removal of larger scale pollutants, such as ethanolamine (from anti-corrosion uses in piping), has also been demonstrated.[151] However, this is the first time that removal of two pharmaceutical micropollutants (17β-estradiol and bisphenol-a) by MFCs has been reported. Although the technology does not provide complete removal of the compounds in question, it can have a very high removal rate at low concentrations, so could clean the wastewater almost completely after a few passes.

These pollutants are usually removed by conventional wastewater treatment processes such as oxidation with chlorine or UV light. However, as discussed in the literature review, removal of these more complex compounds is often incomplete, or partially degraded compounds are left in the water, which have potential to be biologically active in different ways to their parent molecules.[35, 52] As such MFCs could provide a method of almost complete removal, which is a big advantage over current processes. Furthermore, MFCs are a cheap and quick technology which have already been considered for uses involving the monitoring of water quality. Combining this process with treatment of wastewater would be a highly cost-effective and efficient process, in a similar manner to combining water treatment with electricity generation. [11, 12]

5.2.4 – Diclofenac

Results from injection of the biologically active compound diclofenac are shown in Figure 41. These results are significantly less straightforward than for the previous two biologically active compounds. Concentrations below 3 µg mL^{-1} caused a positive response from the MFCs, as with the previous biologically active compounds. However, there was not a linear increasing trend. Instead, apart from the lowest concentration used, the trend was decreasing. This could perhaps suggest that the biologically active compound is metabolised by the bacteria, but that there is an underlying toxic effect, explaining the decreasing current output with increased concentration.

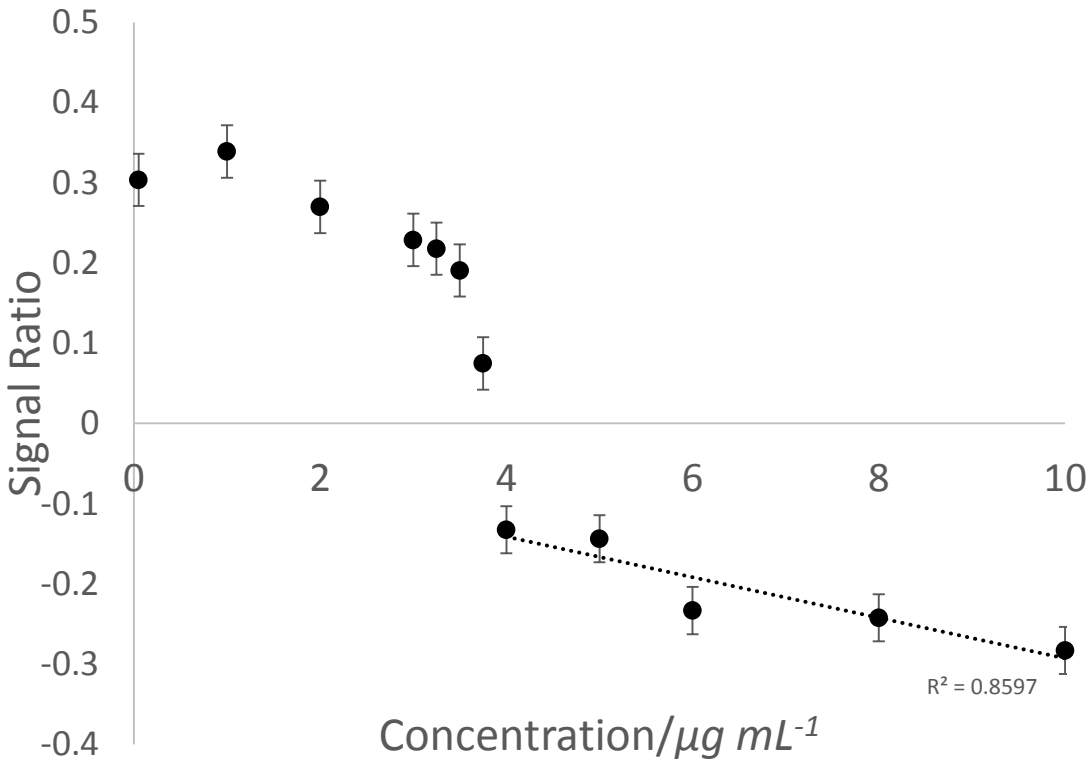

Figure 41 - Effect on the Signal Ratio of 3 MFCs with Various Concentrations of Diclofenac. Injection time 30 mins. The error bars represent 1 s.d. There were 3 replicates.

Concentrations of 4 µg mL^{-1} and above all caused negative responses from the MFCs. That is to say their current output decreased as a result of the biologically active compound injection. This is indicative of a toxic response to the bacteria in the MFC, and is observed for more common biologically active compounds such as metals.[4, 5] The sensitivity was calculated to be 0.0064 18.1 nA μg^{-1} cm^{-3} in the linear region (4-10 µg mL^{-1}) indicating the MFCs are lease sensitive to this biologically active compound. It would appear that diclofenac is non-toxic at concentrations of between 0.05 and 3.75 µg mL^{-1} and below, but is toxic at concentrations of 4 µg mL^{-1} and above.

In the case of diclofenac, LCMS data could not be obtained because another compound in the feed solution is close to the molecular mass of diclofenac and masks its signal. This difficulty could perhaps be overcome by pre-filtering the sample.

It is interesting to note that diclofenac performed differently in the preliminary experiments, causing negative response across the whole concentration range. This could be because different conditions in the new fuel cell design led to different bacterial species surviving in the biofilm, or behaving differently. It is hard to say with any certainty why that might be. Furthermore, the preliminary experiments were carried out with fuel cells which were unreliable, which may mean the data gathered from them is inaccurate.

5.2.5 – Triclosan

Results from injection of the biologically active compound triclosan are shown in Figure 42. All concentrations tested elicited a positive response, with a mostly linear trend. The minimum detectable concentration in this instance was 0.01 µg mL^{-1}. The sensitivity was calculated to be 0.0271 nA μg^{-1} cm^{-3}. This compound is a known antibiotic, often used in children's toys. We would therefore have expected a negative response from the MFCs, as it killed bacteria in the biofilm.

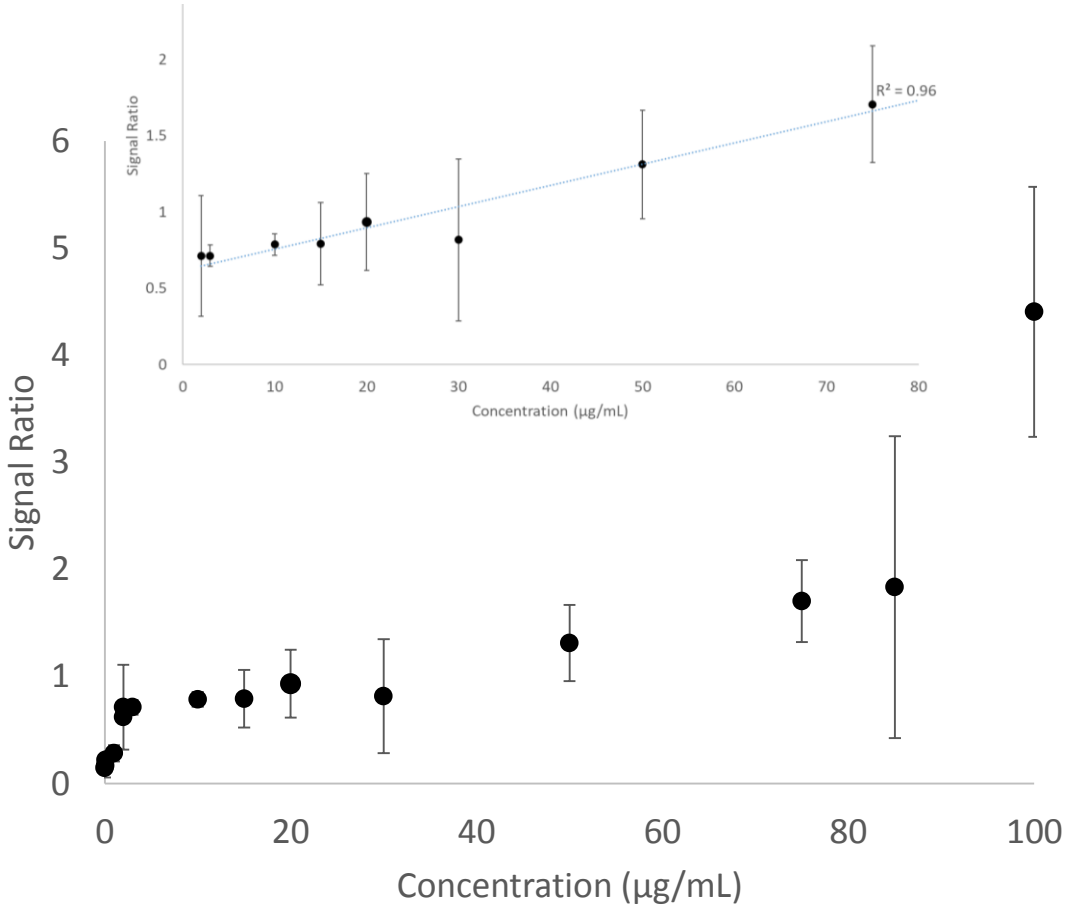

Figure 42 - Effect on the Signal Ratio of 3 MFCs with Various Concentrations of Triclosan. Injection Time 30 mins. The error bars represent 1 s.d. There were 3 replicates.

This, however, was not the case in the range of concentrations tested. The range was increased to include concentrations up to 100 µg mL^{-1}, in the hope of seeing this effect. However, all concentrations still led to positive responses. It was decided not to increase the concentration further as this would have been far outside of the concentrations expected in wastewater, and is therefore not relevant to this investigation. It is surprising that this negative effect was not seen within this large range though, although this could be because the concentrations used may be too low to have an anti-bacterial effect on a large scale. It is difficult to determine if this is the case however, without knowing which species

of bacteria are in the biofilm as the dose which becomes toxic is different depending on the species present. Therefore, it is concluded the bacteria did indeed metabolise the triclosan within this range, and generate extra current output.

LCMS results for triclosan are shown in Figure 43. Removal was greater than 50% for every concentration tested.

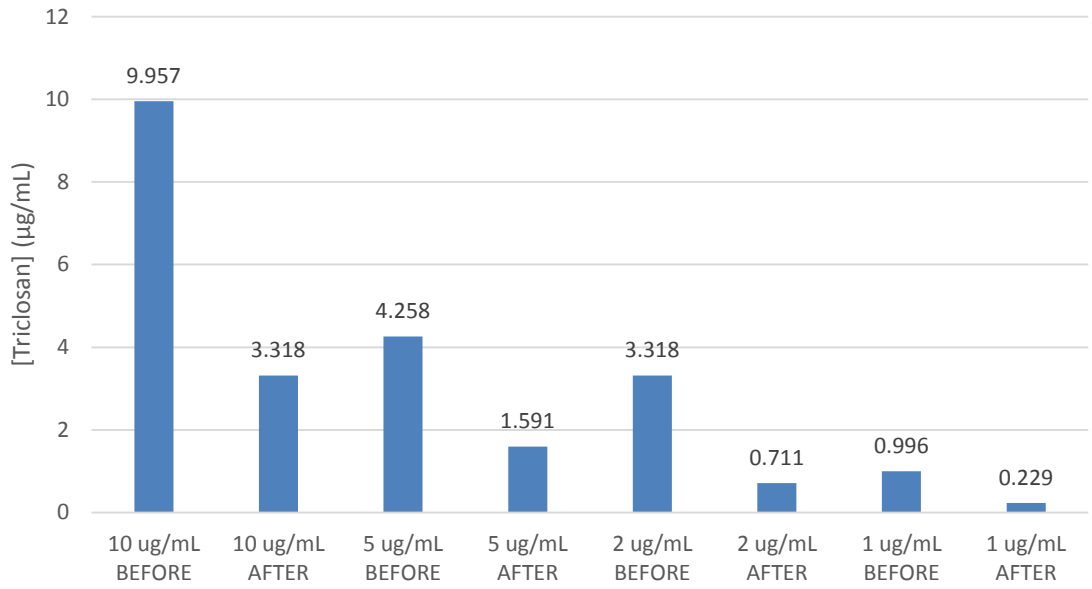

Figure 43 – LCMS Data for Triclosan

However, for this biologically active compound a reduced range of concentrations were tested, due to limited availability of the instrument. Those tested represented a similar range to other biologically active compounds, and still provide a clear general trend. No control samples could be tested to ascertain how much of the removal was due to the same instrument constraints, but the average removal was 71.2%. This is significantly higher than control results for other compounds, which implies that a large part of the triclosan is metabolised.

These results, quite unexpectedly, show that triclosan behaved similarly to bisphenol-a and 17β-estradiol in that it produced positive linear responses across the range of concentrations tested. This implies it is metabolised by the bacteria in the biofilm and thus causes an increased current output. It is also removed at a similar rate, as shown by the LCMS data. It had the lowest detection limit of all the compounds tested and the linearity range was very wide.

Triclosan has been shown to exhibit a broad range of anti-biotic activity to most kinds of bacteria, moulds and yeasts.[136] However, as with all broad-based anti-biotics, it is not effective against all bacteria in the same way. At the concentrations used in this study, and in care products and childrens' toys, it is not acutely toxic to humans, and kills many bacteria. However, to kill other species, a higher dosage would be required, which may cause harm to humans if used. These concentrations were not used in this study, which may explain why the bacteria in the biofilm were able to metabolise it. That is to say, the species in the biofilm were not affected by this low concentration. [152]

5.2.6 – SEM Results

SEM images were collected for a range of concentrations of each of the biologically active compounds tested, at various magnifications. Images shown are all of the same concentration and magnification, for ease of comparison.

Figure 44 reports an SEM image of the anode surface of an MFC exposed to 5 µg mL^{-1} bisphenol-A. Good surface coverage of the electrode is observed, with biofilm covering the most of the anode surface. This coverage is notably better than when the biofilms were exposed to other biologically active molecules. This could be due to increased protein production caused by bisphenol-a and other similar endocrine disruptors. However, the biofilm has not been analysed for its content. Endocrine disruptors have previously been shown to cause certain species of bacteria to extrude more protein into the extracellular matrix, which increases the viscosity of the biofilm. This makes it more resistant to shearing and can lead to a thicker biofilm. [135]

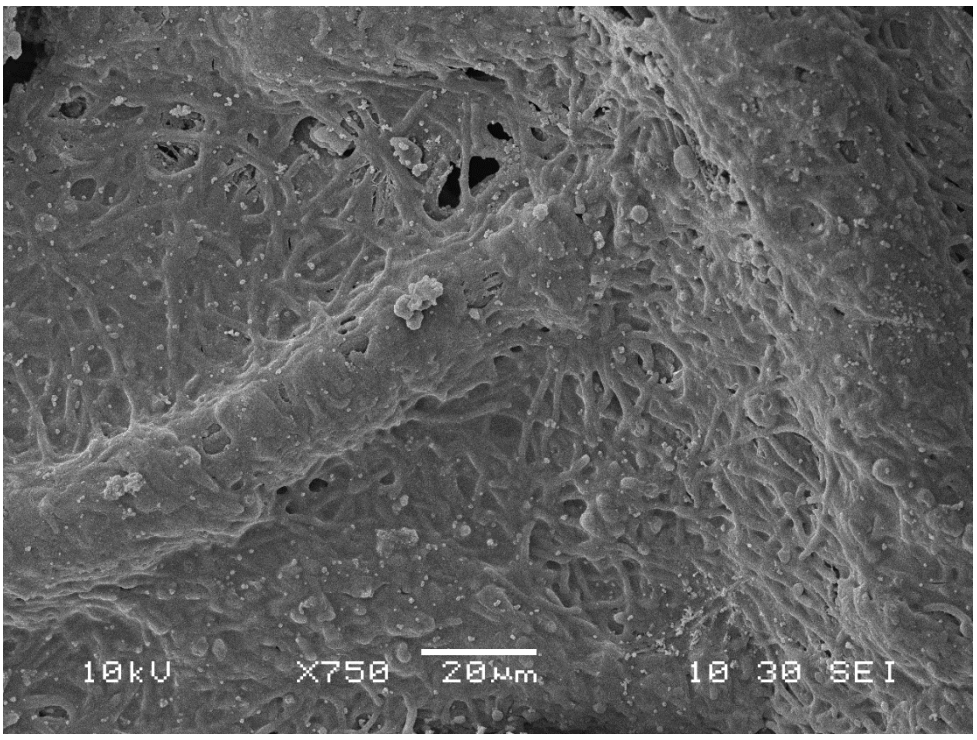

Figure 44 - SEM Image of a Biofilm Exposed to 5 µg mL^{-1} Bisphenol-A. x750 Magnification

An SEM image of the anode surface of an MFC exposed to 5 µg mL^{-1} 17β-estradiol is shown in Figure 45, showing strong biofilm coverage, similar to that of bisphenol-A. Some bacteria are visible on the surface and a good surface coverage is observed, with a small amount of cracking. This furthers the similarities between the two compounds.

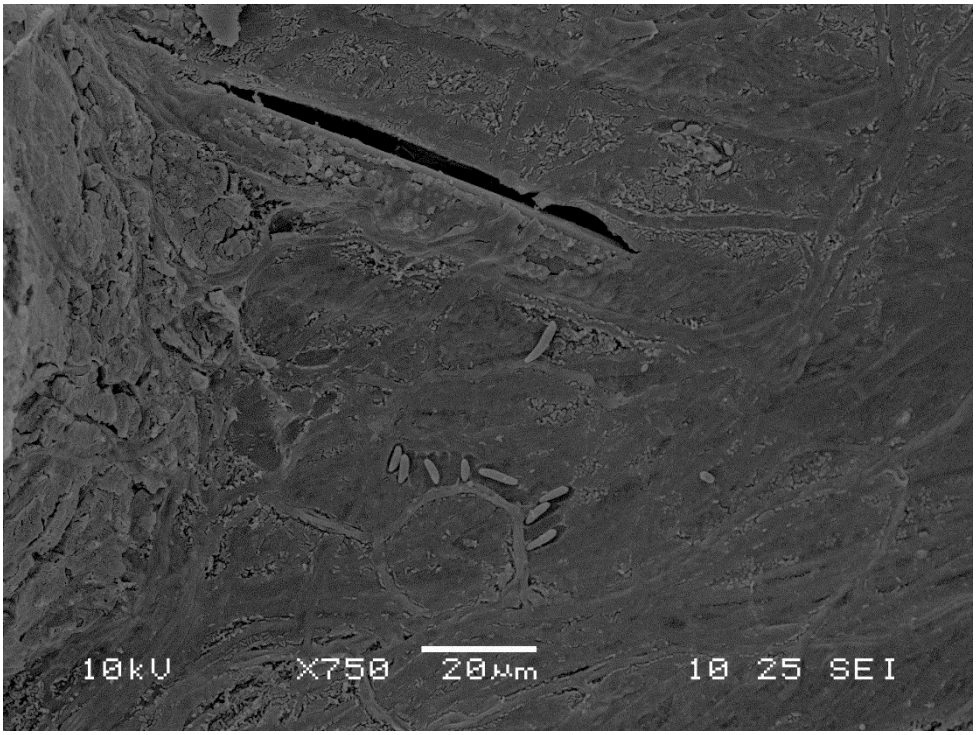

Figure 45 - An SEM Image of a Biofilm Exposed to 5 µg mL^{-1} Estradiol. x750 Magnification

An SEM image of the anode surface of an MFC exposed to 5 µg mL^{-1} diclofenac is shown in Figure 46. Although individual bacteria can be seen, and is some biofilm matter visible, it does not show the same strong coverage of biofilm as with bisphenol-A, perhaps indicating that it was damaged by the presence of the biologically active compound. The strands of the carbon cloth are clearly visible, unlike in the cases of the other biologically active compounds.

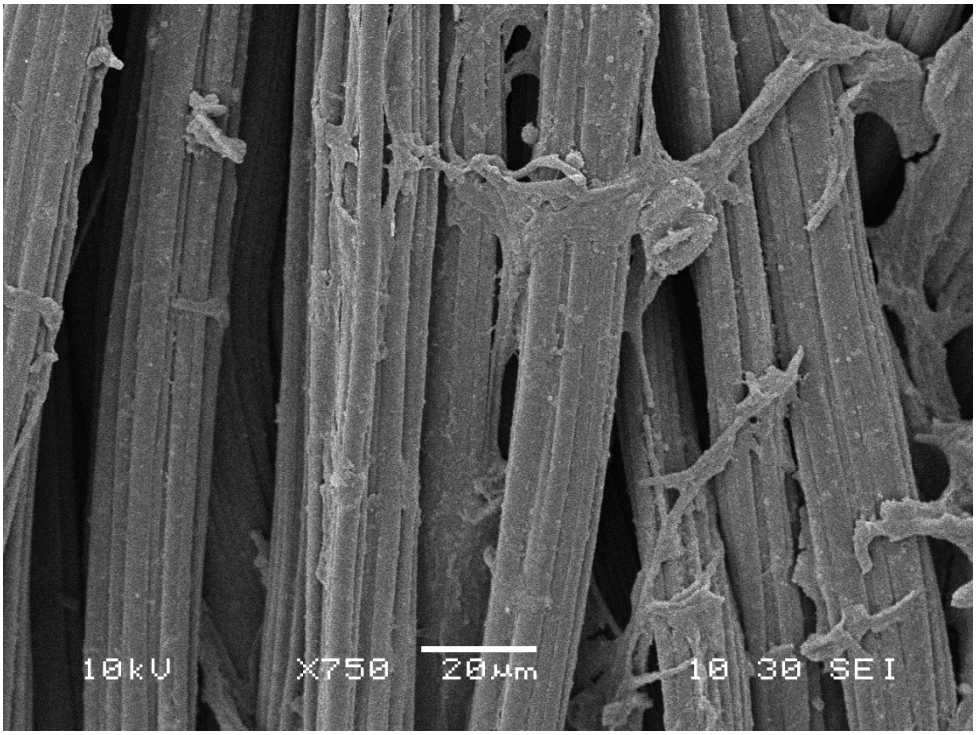

Figure 46 - An SEM Image of a Biofilm Exposed to 5 µg mL^{-1} Diclofenac. x750 Magnification

If the biofilm is indeed damaged by the presence of diclofenac, it may explain why the current output first decreases, then becomes negative with increased exposure to the biologically active compound.

An SEM image of the anode surface of an MFC exposed to 5 µg mL^{-1} triclosan is shown in Figure 47. The biofilm is clearly visible, as are some individual bacteria within that film. However, the biofilm does not offer complete coverage of the surface. This could indicate that triclosan damages this biofilm. However, the coverage is much more complete than is present with diclofenac.

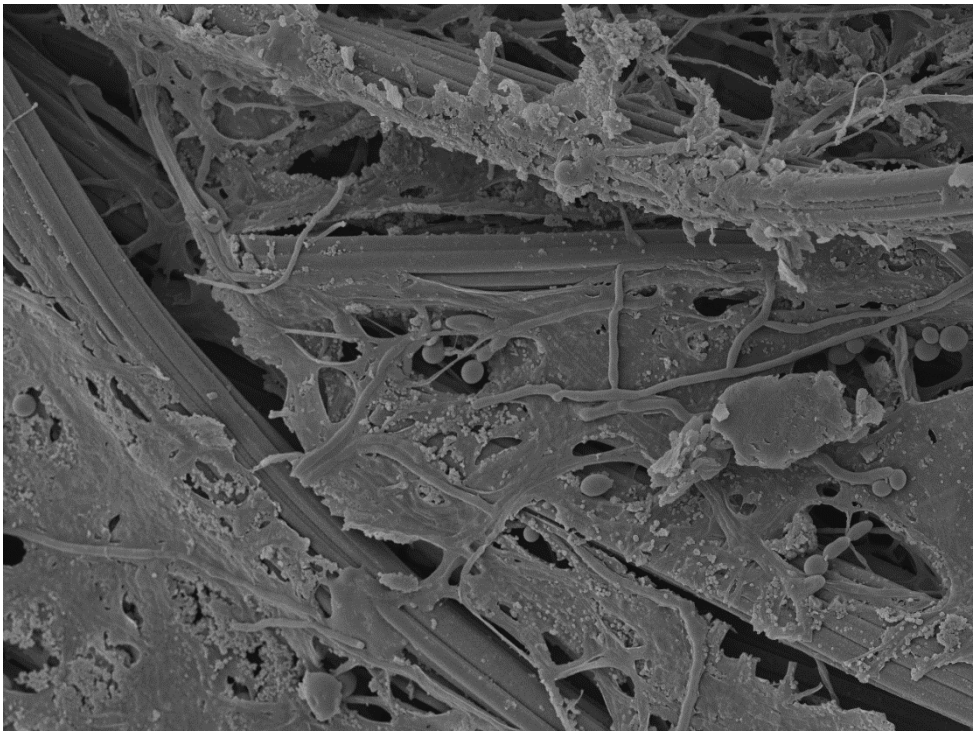

Figure 47 - An SEM Image of a Biofilm Exposed to 5 µg mL^{-1} Triclosan. x750 Magnification

This damage could be caused by the antibacterial properties of triclosan. The fact that the damage is not as extensive as it is when diclofenac is used as the biologically active compound may explain why no negative results were obtained when using diclofenac.

Images exist of a wider range of concentrations, and are summarised in the summary table below.

5.2.7 – Summary of Biologically active compound Injection Results

A summary table of results is shown in Table 10.

Table 10 - Summary Table of Results

Biologically active compound	*Minimum Concentration Detected ($\mu g\ mL^{-1}$)*	*Linearity Range ($\mu g\ mL^{-1}$)*	*R^2 Value*	*Sensitivity ($nA\ \mu g^{-1}\ cm^{-3}$)*	*SEM Imaging*	*Average LCMS Results (% Removal)*
17β-estradiol	0.5	0.5-10	0.9369	18.10	Biofilms exposed to all concentrations exhibited a high surface coverage. This made is difficult to see individual bacteria, but suggested the biofilms were not damaged by the estradiol. Difficult to see individual bacteria, perhaps due to extra protein production caused by endocrine disruptors.	20% by metabolisation, 58% total removal
Bisphenol-A	0.1	0.5-2	0.9604	1.001	All concentrations left in-tact biofilms with high coverage, again indicated they were not damaged by BPA. It was possible to see individual bacteria, indicating extra protein production did not occur.	40% by metabolisation, 94% total removal
Diclofenac	0.05	4-10	0.8597	0.0064	Heavily damaged biofilms with poor coverage. Increasing concentration caused increased damage.	Unable to measure
Triclosan	0.01	0.05-85	0.8762	0.0271	Some damage to biofilms, but less than diclofenac. Damage increased with concentration.	71% total removal, but unable to perform control experiments

As this summary table shows, the MFC-based biosensors were most sensitive to 17β-estradiol. They were least sensitive to diclofenac. This means that an increase of unit concentration causes the largest increase in output of the MFCs. However, the estradiol had the highest lower detection limit, which is interesting. Triclosan could be detected at the lowest limit. 17β-estradiol has been found in wastewaters in the range of 0.2 ng L^{-1} to 78 ng L^{-1}. [134] Bisphenol-A has been found between 0.088 and 11.8 ng L^{-1}. [135] Triclosan has been found in wastewater at levels between 3.8 and 16.6 µg L^{-1}.[136] Diclofenac has been found in the region of 119-1376 ng L^{-1}.[137]

Bisphenol-a and 17β-estradiol are clearly not detected at sufficiently low concentrations by these biosensors. The lower limit of detection was around a factor of 1,000 too high compared to their concentrations in wastewater. A factor of 1,000 is quite a long way off being useful as a sensor in real-life applications. It would require strong advancements in the technology to be able to detect in the necessary range, given the sensitivities of these devices.

However, triclosan was much closer – the lower detection limit was around the same as the highest concentration detected in wastewater in the study mentioned previously. This is promising as it demonstrates that with only a little enhancement, these biosensors could detect within a range useful for the treatment of wastewater. The sensors were also able to detecting diclofenac in close to sufficient concentrations, only being around a factor of 10 out from the range of concentrations found in that study. Again, this is quite promising as there are no technologies with this low level of cost which can detect these compounds at this concentration currently available. Most have to be detected by some form of chromatography, followed by mass spectrometry, which is ultimately very expensive and much slower than the results gained by these MFCs.

LCMS results show further promise, as very significant quantities of all of the biologically active compounds we could test were removed by flow through the MFCs. Not all of this removal was due to metabolisation by the biofilm, but significant parts were. This could present a very cheap and quick tool for removing these compounds from wastewater, and merits further investigation in later experiments. It was particularly impressive that and average 94% of bisphenol-a could be removed by just one MFC.

SEM results provided a potential insight into what was happening within the biofilm itself. Biofilms exposed to bisphenol-a showed good biofilm coverage at all concentrations and individual bacteria were easily seen. This implied they were not damaged by the biologically active compound. Those exposed to 17β-estradiol were also undamaged, but individual bacteria were not visible, and coverage seemed to increase with concentration too. This implies that the estradiol had an effect which increased the production of extracellular proteins, covering the bacteria in a slimy layer, making seeing individual bacteria difficult. [135, 139]

Conversely, biofilms exposed to increasing concentrations of diclofenac seemed to have steadily more damage – the coverage was worse with increasing concentration of the biologically active compound. This implies that it damages the biofilm, and has a toxic effect

on the bacteria. This is mirrored by the negative effect the biologically active compound has on the current output of the MFCs at higher concentrations and thus, the two may be linked. Triclosan also seemed to damage the biofilm with increasing concentration, which is not surprising considering its antibacterial properties, but this did not seem to affect the current output of the MFCs in the same was as diclofenac. The reason for this is unclear, but it could perhaps be that triclosan at these low doses inhibits production of extracellular proteins, but does not yet have a toxic effect on the bacteria, or inhibit the oxidation of the fuel source. Indeed, at the concentrations used in this study, triclosan is not considered toxic to bacteria. [22]

This kind of organic micropollutant is difficult and expensive to detect in wastewater. It is not regularly monitored, save for sampling every months and then detection *via* techniques such as LC-MS. [57, 61] This is a very time-consuming and expensive method and does not provide real-time information on the concentration of these micropollutants or how well they are being removed by treatment processes on a day to day basis.

MFCs have been shown to be effective at providing real-time or at least very quick measurements of toxicity arising from toxic metals or highly toxic organic compounds on a timescale of minutes. [2, 11, 15, 17, 37] However, for the first time, this work reports the detection of biologically active organic micropollutants and on a similar timescale of around 30 minutes – although a signal can be seen even earlier than that. This is a large improvement on sampling-based techniques and may help monitor water treatment processes in real time. Furthermore, it does not require expensive laboratory equipment (LC-MS) or offsite laboratories to perform the analysis, leading to large cost savings.

Other real-time methods of monitoring the presence of organic molecules do exist. One such example is optical spectroscopy. Using light in the IR, near-IR, and UV regions, absorbance values can be obtained at specific wavelengths which correspond to certain features of molecules. However, this technique requires some initially expensive equipment, and is complicated by the presence of many similar and overlapping absorbances arising from similar molecules, limiting its practical use. [40, 62]

Another alternative is traditional electrochemical biosensors. These work by directly measuring the concentration of a micropollutant based on the electrical current produced during a redox process taking place on a surface electrode. They are quick and highly sensitive, but are usually very selective, detecting either a single compound or group of compounds. However, they have been successfully used to monitor the concentration of

herbicides in wastewater by monitoring the redox processes associated with respiration rate of microalgae. [153] This selectivity gives MFCs an advantage in terms of detecting a wider range of compounds, however electrochemical biosensors are generally more sensitive. MFCs need to overcome this barrier of sensitivity in order to compete, and this may be addressed by control of anode potential. [13]

5.3 – Long Term Exposure Experiments

Resistance to biologically active compounds and pharmaceuticals is a well-documented effect in many organisms, including bacteria. [22] It was decided to perform experiments in order to ascertain whether this effect was noted when operating the MFCs over longer periods in order to ascertain their suitability for long-term use as biosensors for biologically active compounds.

There were two types of long-term experiment performed with respect to each biologically active compound. The first was long-term exposure to the biologically active compound at a single concentration (0.05 µg mL^{-1}) to attempt to see if there were any effects, such as resistance to the biologically active compound, which developed over time.

The second type of experiment exposed the MFCs to 4 different concentrations of the same biologically active compound, in order to determine whether the MFCs could be used as a sensor to monitor the concentration of the biologically active compound over a period of time. In both cases, the cells were fed with clean feed before injection started.

In all cases, the traces below are formed by taking a reading every minute. The lines are point to point.

5.3.1 – Diclofenac

The response of the MFCs exposed to 0.05 µg mL^{-1} diclofenac is shown in Figure 48.

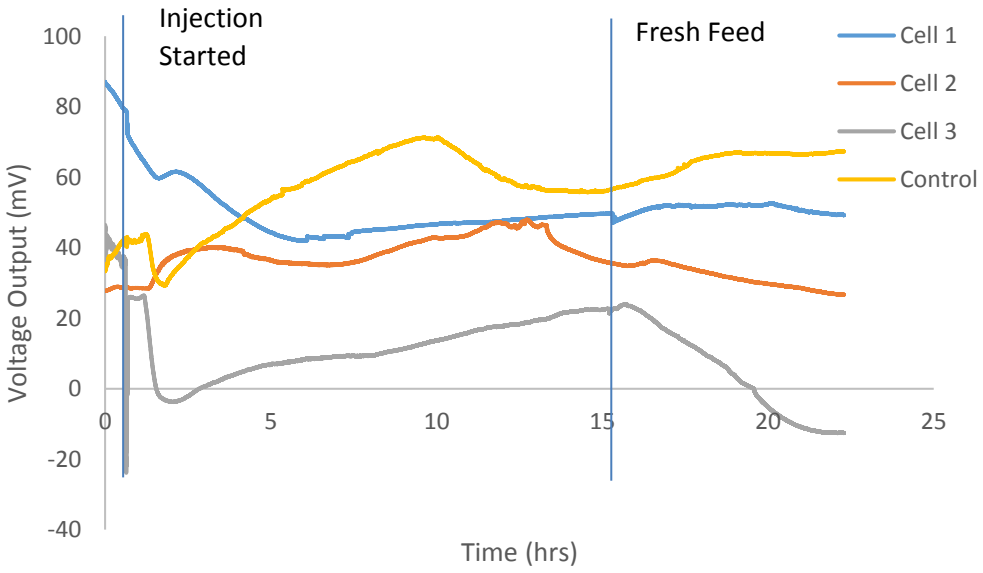

Figure 48 - Response of 3 MFCs Exposed to 0.05 µg mL^{-1} Diclofenac Over Extended Periods

Once the diclofenac was injected, cells 1 and 2 experienced a positive response to the biologically active compound, in line with short term experiments previously performed. Cell

3, however, experienced a strong negative response, which was more typical for higher concentrations of diclofenac. The control cell (which was not injected with diclofenac) also experienced a negative response, but to a much lesser extent.

Cells 1 and 2 then went on to show fairly stable outputs for the remainder of the experiment, which could imply that long-term, this longer term diclofenac exposure has minimal impact on the output of the MFCs. However, cell 3 recovered from its initial negative response, particularly after the first injection of fresh feed. The second injection of fresh feed then seemed to cause a strong negative response again. The control also seemed to suffer some variance, but it is not clear why.

The experiment was repeated, but despite successive attempts, no clearer results were obtained. It was therefore decided not to perform the experiment using multiple concentrations, as it would be difficult to elucidate the meaning of any results, with only this for a background.

5.3.2 – Bisphenol-A

The response of the MFCs exposed to 0.05 µg mL^{-1} BPA is shown in Figure 49.

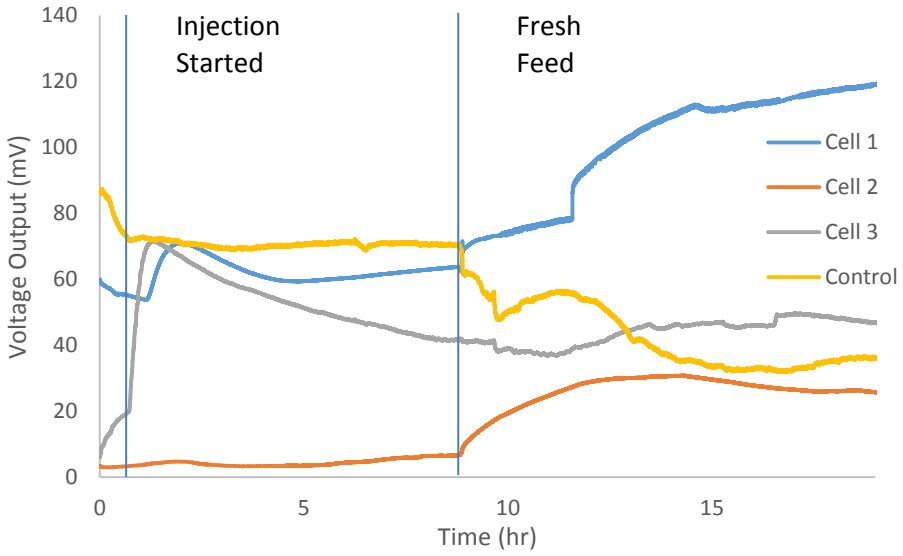

Figure 49 - Response of 3 MFCs Exposed to 0.05 µg mL^{-1} BPA Over Extended Periods

All cells injected with biologically active compound initially showed a positive response, although it was much smaller in cell 2 (perhaps because of the initial low baseline current). This is in line with short-term experiments. The outputs then decreased and stabilised over time. Again, those cells injected with biologically active compound experienced a second positive response when new feed was added (at the same concentration as previously). The response of cells 1 and 3 were delayed compared to that of cell 2. This could be because of

uneven flow rates between the cells. Although the pump speed is constant, pressure drops and blockages could cause the effective rate to slow in some cells. Flow rate is not monitored continuously.

It is unclear why providing fresh feed to the cells after injection of the BPA should illicit such a strong response. The output voltage of each of the cells was much greater than before biologically active compound injection commenced. Although the biologically active compound is known to illicit a positive response, the fresh feed was identical to the feed used beforehand. It is possible that over time, some of the carbon source in the feed is degraded under ambient conditions and that using fresh feed replaced that which had been degraded. However, this is unlikely given the relative stability of the compounds involved.

Results from the injection of various concentrations of BPA over an extended time period are shown in Figure 50.

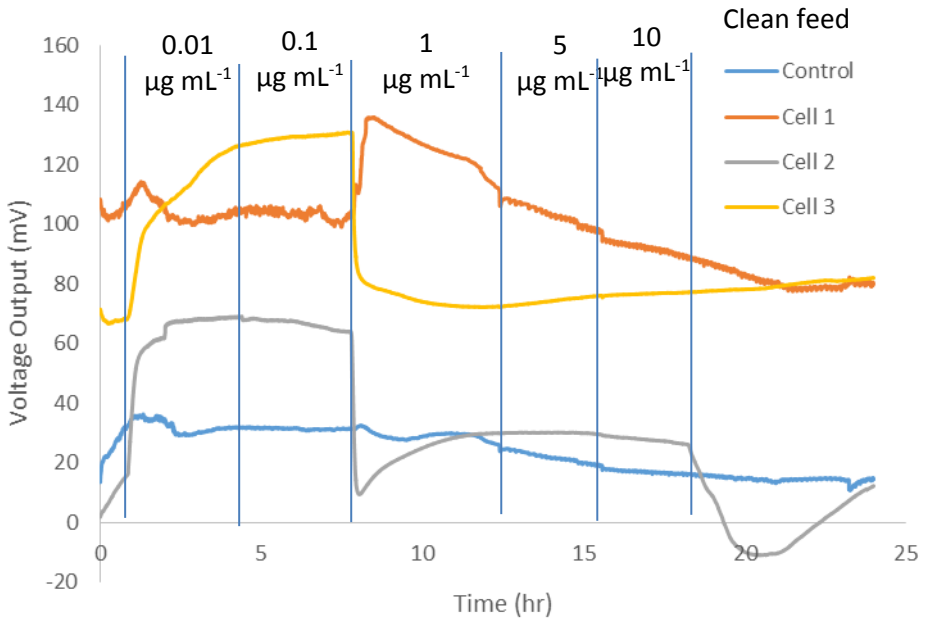

Figure 50 - Response of 3 MFCs Exposed to Various Concentrations of BPA Over Extended Periods

On injection of the first concentration, the cells injected with biologically active compound exhibit a positive response, as expected. On the next concentration increase, there is little response from the cells. Perhaps because the difference is not enough to detect easily – 0.01 µg mL^{-1} is below the detection limit demonstrated in short-term experiments. However, here we see a response, perhaps due to the larger timescale.

The injection of the 3rd concentration causes a strong negative response in two of the cells, but a strong positive response in the other. This is unexpected, because this biologically active compound has always elicited positive responses in all previous tests. Perhaps this change is due to the longer-term exposure to BPA at gradually increasing concentrations, causing the biologically active compound to be more harmful to the biofilms than in previous cases. However, no such effect was observed with exposure to BPA at continuous low levels.

Further concentration increases caused no further response from the MFCs. It is possibly that the biofilms had already been damaged by earlier concentrations, and were possibly too damaged to be viable enough to detect further changes, which has been exhibited with micropollutants such as formaldehyde. [10] These results show that the MFCs do seem to detect initial changes in concentration of BPA, after such a long exposure to the biologically active compound, they eventually become unable to detect any change. This could be perhaps be because they have been damaged by the exposure. Alternatively, the bacteria in the biofilm could have become resistant to its presence and its presence no longer causes a significant response. The baseline currents returned to very similar values to their initial levels after 12 hours.

5.3.3 – 17β-Estradiol

The response of the MFCs exposed to 0.05 µg mL^{-1} estradiol is shown in Figure 51.

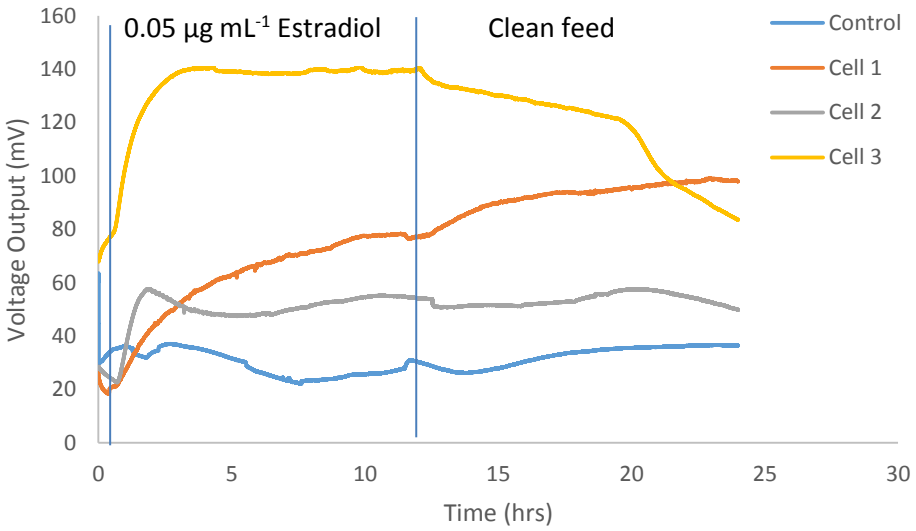

Figure 51 - Response of 3 MFCs Exposed to 0.05 µg mL^{-1} Estradiol Over Extended Periods

Results from this experiment show an initial response to the biologically active compound in line with what was expected – the biologically active compound caused a positive response in all fuel cells injected. The control showed no such response. In cells 2 and 3, the value continued to increase until a plateau was reached. In cell 2, a small dip was observed before that plateau was reached. No large change in the output voltage between initial output and the output after returning to clean feed solution, unlike the results from injection of BPA. This is more expected, as if the solutions are identical, there should be no reason that the output of the MFCs would change.

Cell 3 exhibits a dip in performance to the end of the experiment, which is not exhibited by the other cells. This could be an effect of the continuous exposure, but if that were the case, it would be expected to be evident in all the cells. Since this is not the case, it is more likely the result of a reduced flow rate, perhaps caused by a partial blockage.

Results from the injection of various concentrations of BPA over an extended time period are shown in Figure 52.

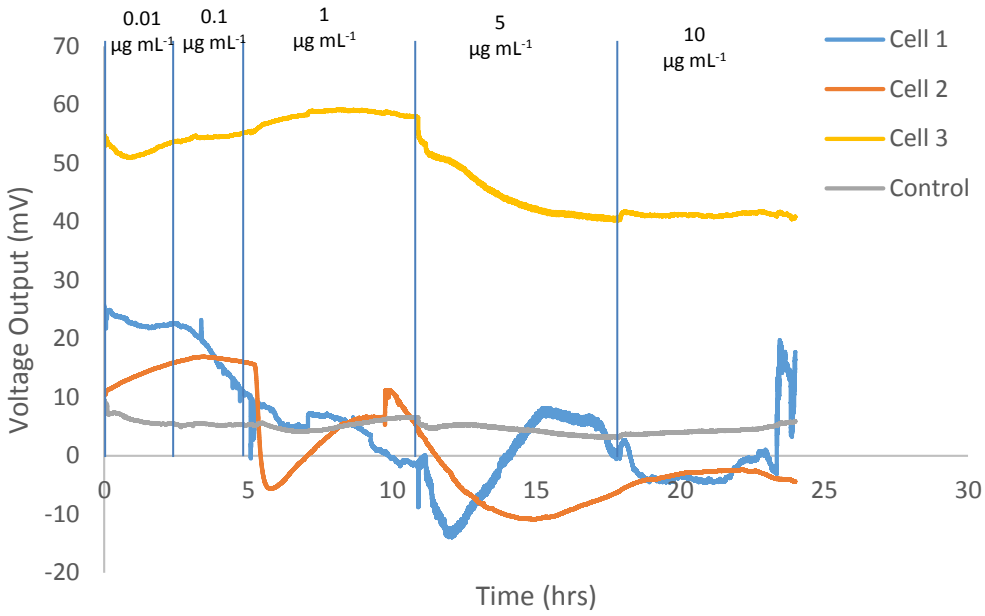

Figure 52 - Response of 3 MFCs Exposed to Various Concentrations of Estradiol Over Extended Periods

Injection of the first concentration of biologically active compound exhibited no real response from the MFCs – very slight changes were visible, but nothing conclusive was observed. This is not surprising, as this concentration was below the detection limit observed in previous experiments. The 2nd concentration began to yield results in that negative changes were observed in cells 1 and to a lesser extent in 2. The 3rd concentration (1 µg mL^{-1}) yielded a strong negative response in cell 2, driving the cell into negative output. Cell 1 also became negative at this concentration, but the change was less sudden.

At 5 µg mL^{-1}, a large negative response was seen in both cell 2 and cell 1. Cell 1 was already negative at this time. Cell 2 also became negative at this concentration. The highest concentration caused no real response in two of the MFCs, but Cell 2 demonstrated a small increase followed by a negative response.

These results are similar to those for BPA in that prolonged exposure to increasing levels of biologically active compound seems to cause negative effects in the MFCs which are not exhibited in either short term experiments or in long term experiments at a constant low concentration. This is an effect which is well-known in toxicology. Many compounds do not exhibit a toxic behaviour in low concentrations in the short term, but often do in longer term experiments. This has indeed been noted for similar compounds. [22, 49, 57] Results from this biologically active compound, however, were much clearer in that all cells experienced negative responses at higher concentrations. Furthermore at least one cell registered a change in concentration had occurred in each case, which was not true for BPA. This demonstrates that the MFCs can monitor a change has taken place over this long time

period, however quantifying the change would be difficult because not all cells responded in the same way to the same concentrations. Although a toxic effect is seen with each increase in concentration, the effects of long term exposure to increasing levels are poorly understood and more work would be needed to make this technology useful.

5.3.4 – Triclosan

The response of the MFCs exposed to 0.05 µg mL^{-1} triclosan is shown in Figure 53.

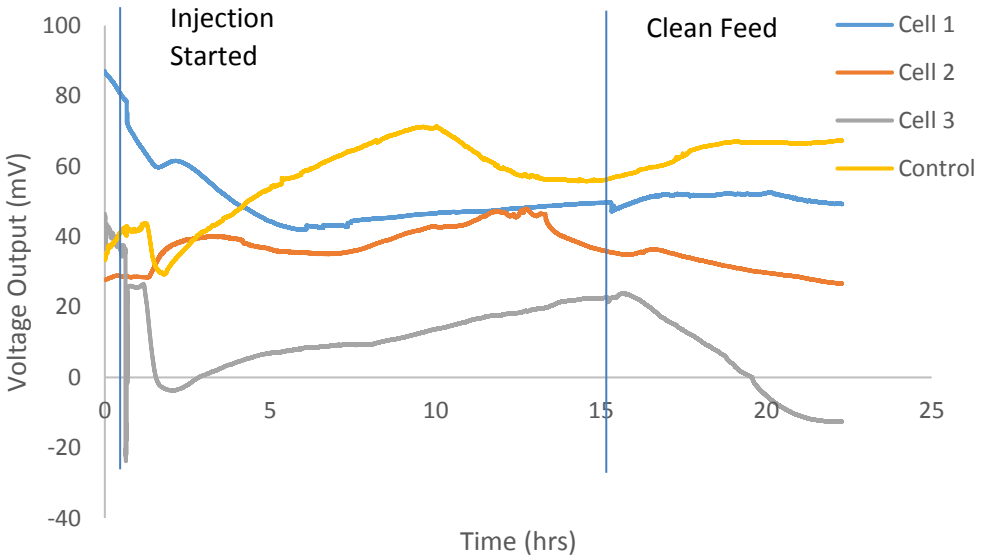

Figure 53 - Response of 3 MFCs Exposed to 0.05 µg mL^{-1} Triclosan Over Extended Periods

A similar effect to other biologically active compounds is observed with triclosan. After injection, an initial positive response is noted in all of the cells injected, followed by a decrease until a plateau is reached. No large change was noticeable upon the changing of the feed solution. These results are in line with what would be expected and previous short-term tests. Both cells 8 and 15 levelled out at values which were much lower than their initial baseline current – this could be indicative of a toxic effect of the long-term exposure to even a low level of triclosan. This would be unsurprising given its antibacterial properties.

Results from the injection of various concentrations of BPA over an extended time period are shown in Figure 54.

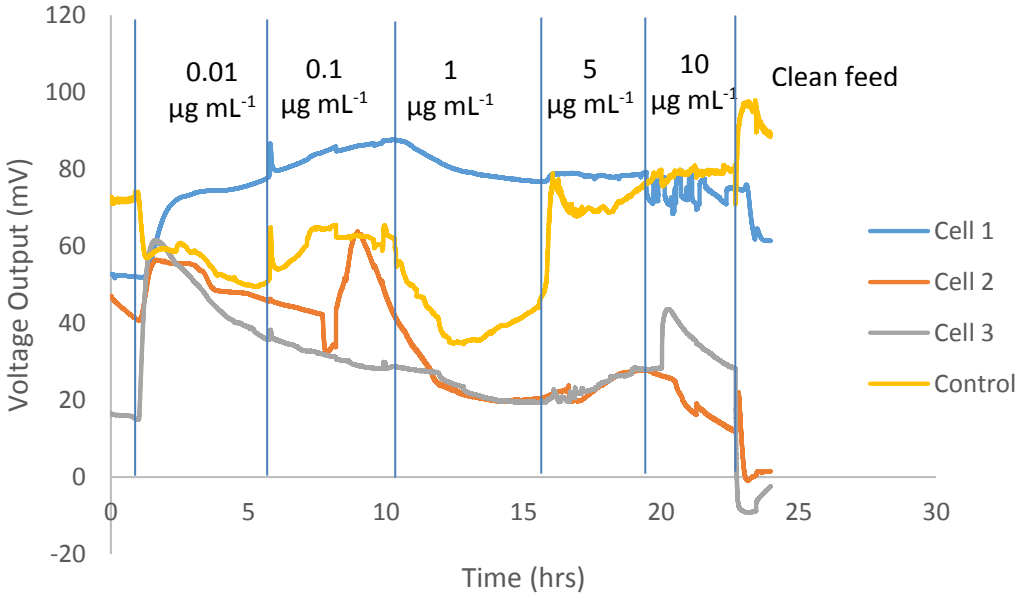

Figure 54 - Response of 3 MFCs Exposed to Various Concentrations of Triclosan Over Extended Periods

The lowest concentration caused significant increases in all 3 of the cells injected, which then levelled off after time. Injection of the next biologically active compound (0.1 µg mL^{-1}) saw a small and slow positive response in 2 of the cells, as voltage steadily increased, although a delayed large positive response was recorded by cell 2, similar to the initial response. This could perhaps indicate a problem with the feeding of the cells, as it would be expected that all the cells respond together, and in the same way. Furthermore, a small spike was observed in some of the cells at this concentration, which is consistent with a small amount of air getting into the system. This could be responsible for that peak.

Injection of 1 µg mL^{-1} caused a steady negative response in all of the cells, although it was much less pronounced in cell 3. However, the control cell also suffered a large negative response here, so perhaps something else was occurring, such as a temperature change. Injection of 5 µg mL^{-1} didn't cause much response from any of the cells, but 10 µg mL^{-1} demonstrated a positive response in cell 3, and a negative response in cell 2.

Again, responses are noted for some concentrations, and increasing concentration causes some more negative responses than previously. However, this demonstrates, as with results from other biologically active compounds, the MFCs can detect concentration changes of these biologically active compounds over a long time period, but the response is often

unclear meaning that the concentration would be unquantifiable. The inconsistency of response in all cells and the fact that the magnitude of changes is often not correlated with the magnitude of the concentration injected mean that these cells are not really suited to being long term, on-line sensors for continuous monitoring of the presence of any of these biologically active compounds.

There may be some long-term exposure effects which are as yet not understood, which are demonstrated by the fact that long-term exposure to one concentration of biologically active compound often seems to yield a different baseline current to the baseline current obtained before injection. They are, however, still suited to short shock-based testing for rapid, cheap detection of these compounds.

Any long term sensor would need to be reliable and consistent – results from different time frames would need to be comparable. [1, 63, 65] Unfortunately, these results do not provide that stability over time. However, sudden changes in output are noted when biologically active compounds are injected, meaning they are still suitable for short-term "shock" tests. [17]

5.4 – Control of Anode Potential

A problem often encountered in this field of work is that results obtained simultaneously from identical MFCs were not always comparable as the baseline currents of different MFCs were not always the same. This could be due to a variety of factors, such as uneven biofilm growth at the anode, small differences in flow rate as blockages occur and then break apart, differences in biofilm composition, etc.

In all cases, the traces below are formed by taking a reading every 10 seconds. The lines are point to point.

5.3.1 – Bisphenol-A

Results from the injection of 0.5 µg mL^{-1} BPA are shown in Figure 55.

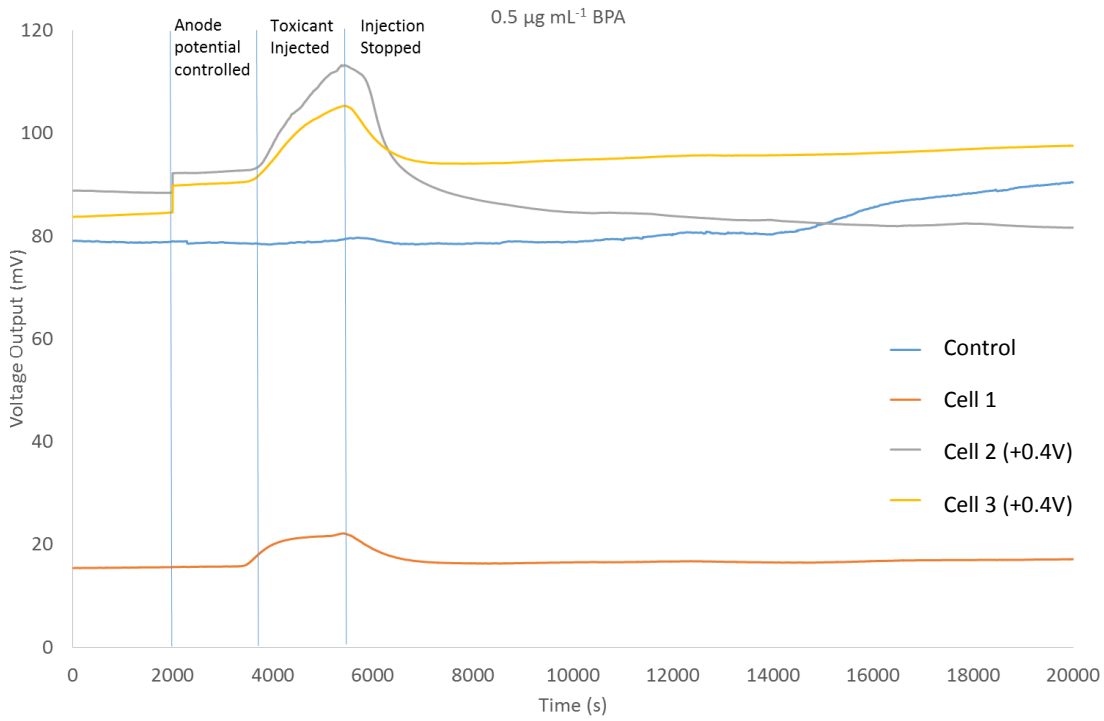

Figure 55 - Injection of 0.5 µg mL^{-1} BPA with 2 cells under anode control

As shown, injection of BPA causes a rise in voltage output of the cell, in all those injected with it. This is in line with previous experiments when cells were not under anode control. No such rise is noted in the control cell, as expected. What is notable about these results is that when the anode potential is applied, it causes those to cells to converge in terms of their voltage output, at a higher value than previously. The change is very quick. They remain stable after this, until the micropollutant is injected. The two cells under anode control exhibited similar shapes of the peaks during injection, whilst the cell without anode control

showed different peak shapes. Similar trends were observed for other concentrations of BPA.

Results from the injection of 2 µg mL^{-1} BPA are shown in Figure 56, and exhibited very similar trends.

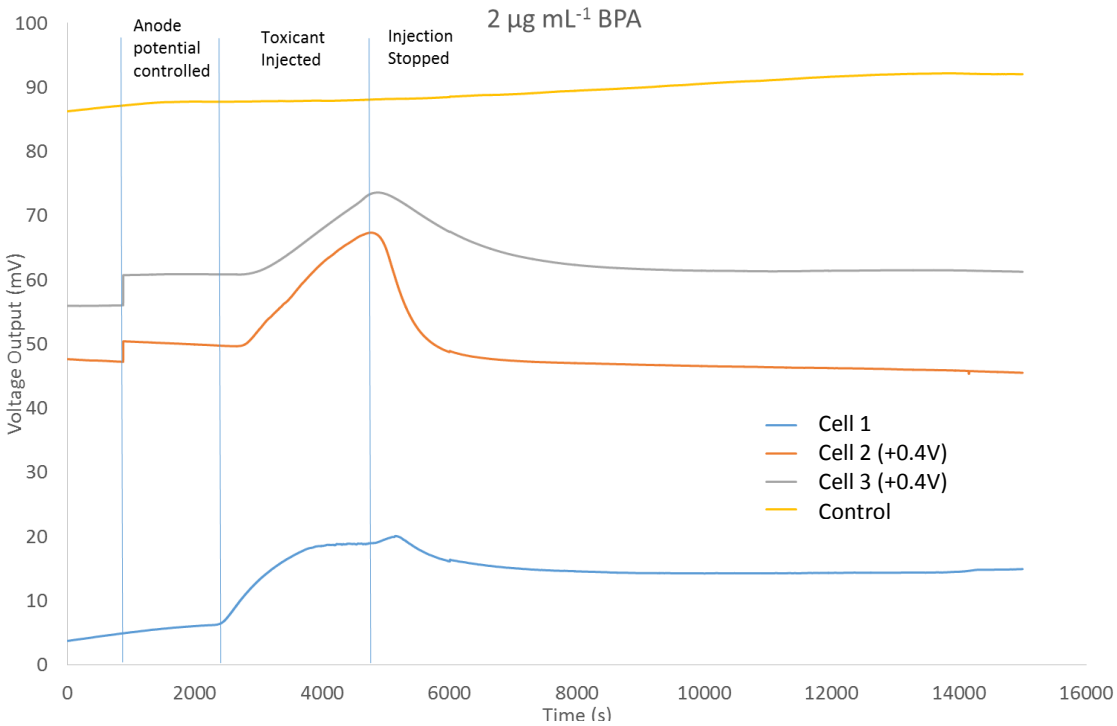

Figure 56 – Injection of 2 µg mL^{-1} BPA with 2 cells under anode control

The convergence of the two MFCs with anode control was less pronounced in this case, but the peak shapes were similar. The magnitude of the peaks was again quite similar in those cells which were under anode control.

Figure 57 shows the results from injection of 5 µg mL^{-1} BPA.

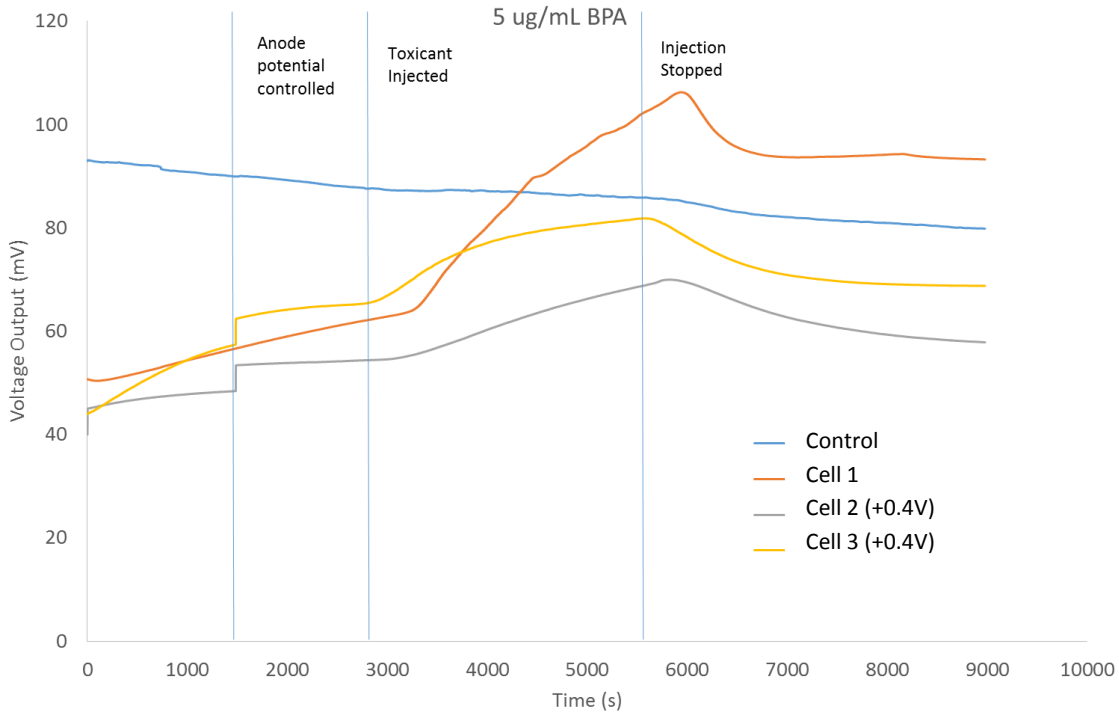

Figure 57 – Injection of 5 µg mL^{-1} BPA with 2 cells under anode control

Results were, broadly speaking, similar again. However, for this concentration it is clear the magnitude of the peaks is much larger.

Figure 58 shows the results from injection of the highest concentration, 10 µg mL^{-1} BPA. These results are show the most difference in peak shape between the two cells under anode control, but the difference is only small. The shape is still broadly the same, so the results are still considered valid.

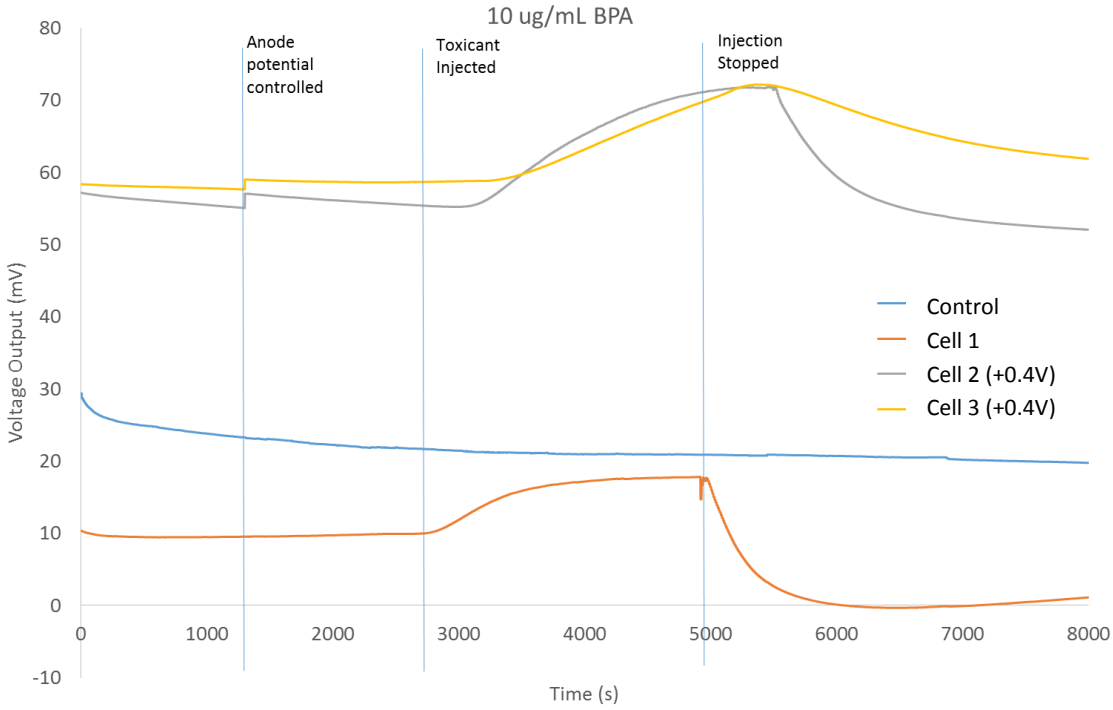

Figure 58 – Injection of 10 µg mL^{-1} BPA with 2 cells under anode control

However, the magnitude of the peaks is very similar and the baseline from which they operate is very similar, so it is thought that these results are still valid. It is notable that throughout these experiments, it is a common trend that the cells under anode control return to a baseline operating current after micropollutant injection which is much closer to their baseline current before micropollutant injection than their counterparts not under anode control. This is because of the extra stability the overpotential provides in terms of the energy produced per electron over the course of each experiment.

A graph of the signal ratio against concentration of BPA in the cells under anode control is shown in Figure 59. The trend displayed is not linear, going against the trend observed for this concentration range without anode control. This could be a kinetic effect of the anode control, not present when the cells are under the control of external resistance.

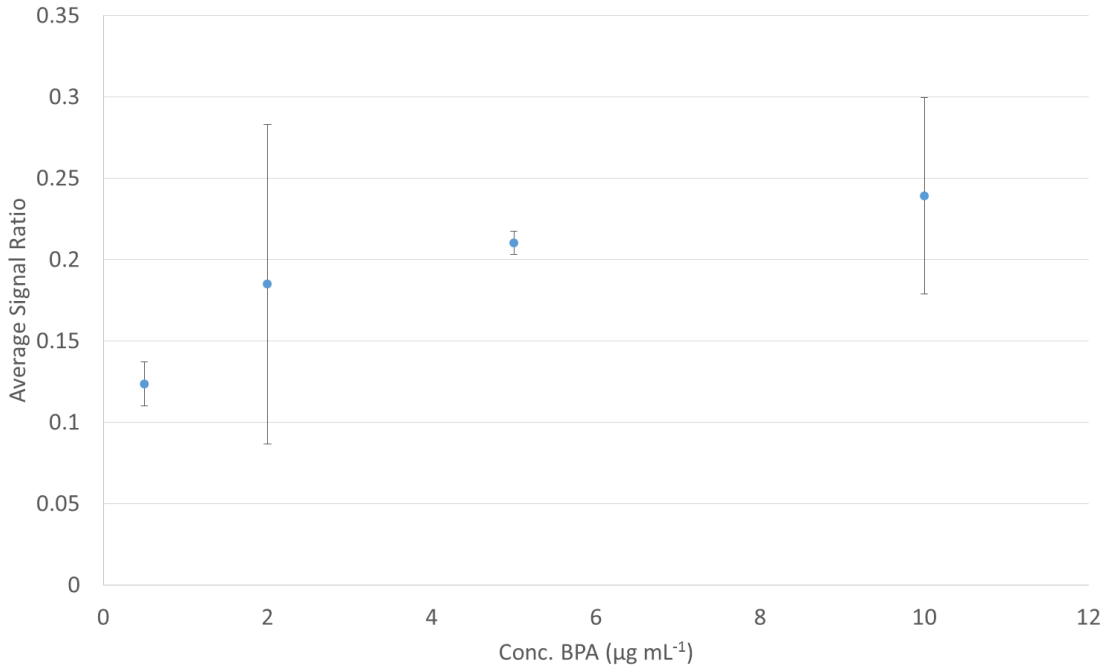

Figure 59 – Overall Results from BPA

However, these results should be interpreted with some caution, as large error bars are present for two of the concentrations. This is probably due to the low number of replicates. The sensitivity is calculated as 0.164 m nA μg^{-1}. This shows the MFC-based biosensors are much less sensitive to BPA under the conditions of anode control, but this is within the range of sensitivities displayed towards other micropollutants without anode control, and is thus not a cause for concern.

5.3.2 - 17β-Estradiol

Broadly speaking, the results for 17β-Estradiol were very similar to those for BPA. The control of anode potential caused the convergence and stabilisation of the output of the MFCs concerned. Peak shapes were very similar in shape and magnitude for those cells under anode control, and the cell not under anode control was often different. The control exhibited no response. In most cases, the cells under anode control were even closer together in terms of output than in the case of BPA. Results are shown in Figure 60 to Figure 63.

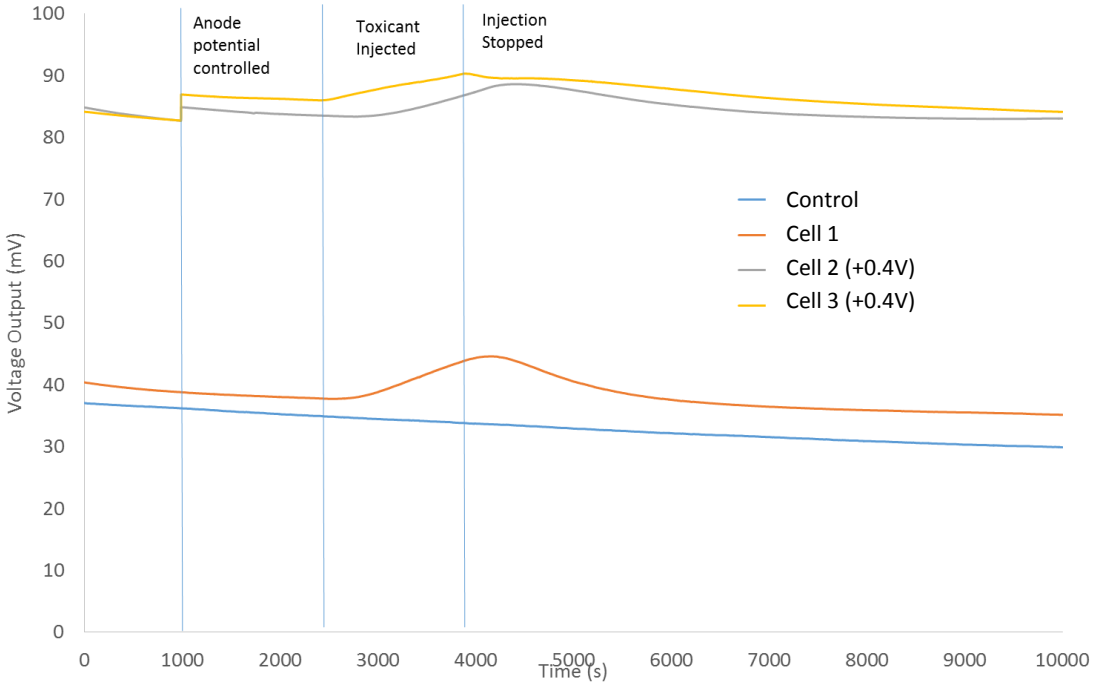

Figure 60 - Injection of 0.5 µg mL^{-1} Estradiol with 2 cells under anode control

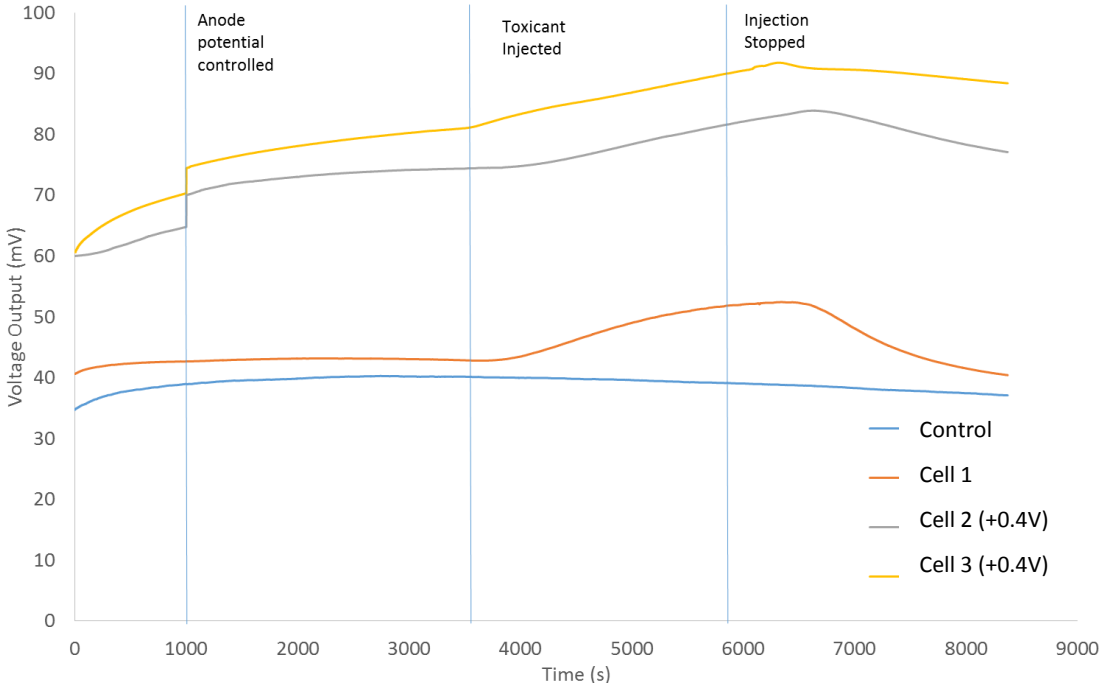

Figure 61 - Injection of 2 µg mL^{-1} Estradiol with 2 cells under anode control

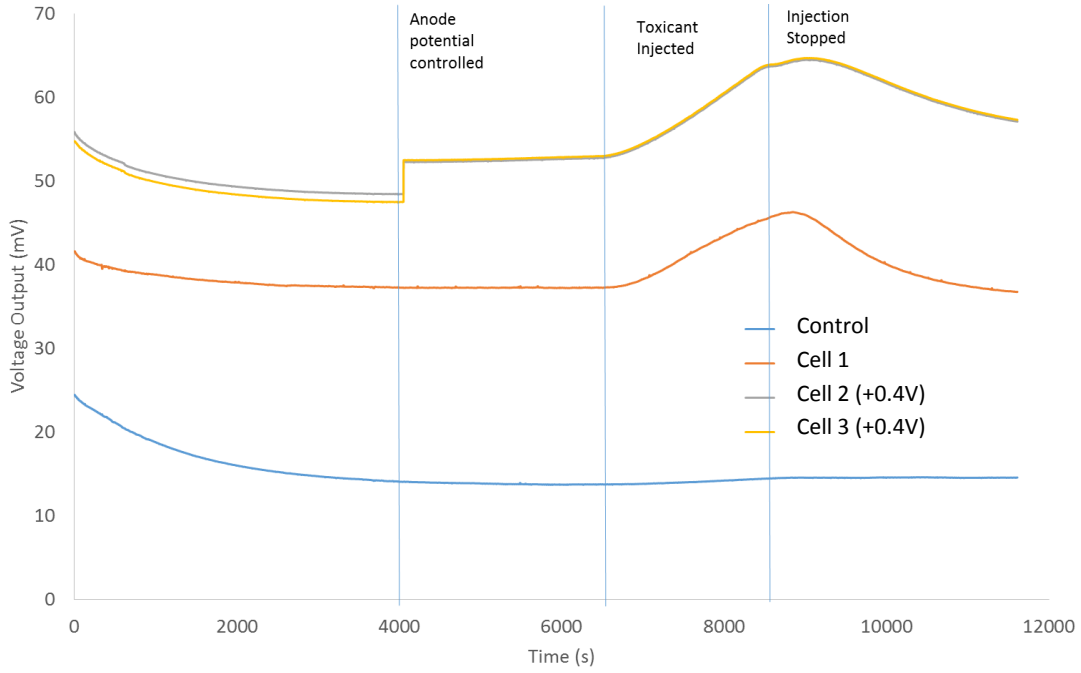

Figure 62 - Injection of 5 µg mL^{-1} Estradiol with 2 cells under anode control

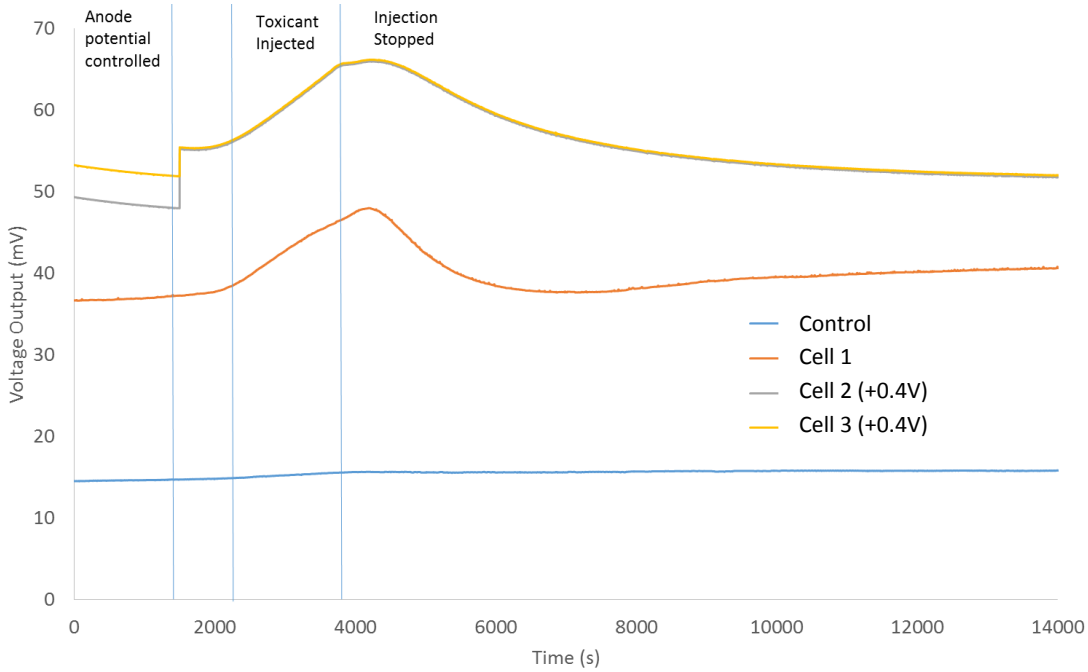

Figure 63 - Injection of 10 µg mL^{-1} Estradiol with 2 cells under anode control

As is evident from the results, injection of 17β-Estradiol under control of anode potential yields results which are incredibly similar to each other in all cases. This can be attributed to the extra stabilisation provided by the control of anode potential. This clearly demonstrates that such control is a very useful tool in terms of making results from different (but identical) MFCs comparable to each other, reducing variability, thus making a much more useful biosensor.

A graph of the signal ratio against concentration of estradiol in the cells under anode control is shown in Figure 64. The trend displayed is close to linear, with one point deviating slightly. This is in keeping with the trend observed for this concentration range without anode control in previous experiments.

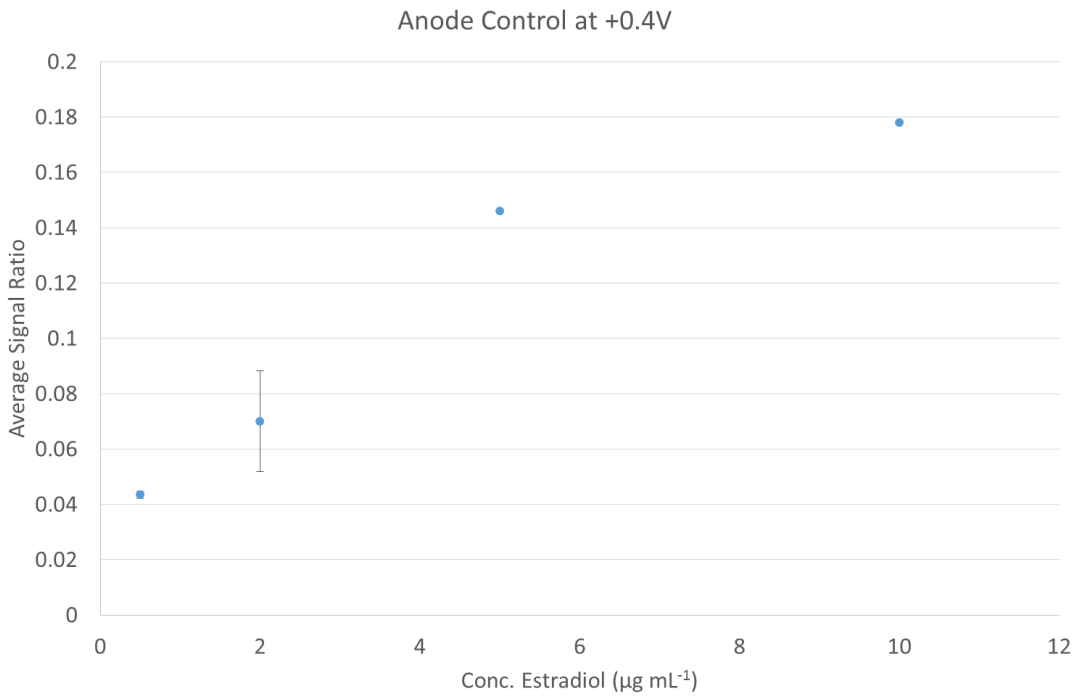

Figure 64 – Overall Results from Estradiol

The error bars with this micropollutant are much smaller, due to the greatly reduced variability in results shown by this micropollutant. However, the number of replicates is still fairly low, so the results should be treated with some caution. The sensitivity was calculated to be 0.689 m nA μg^{-1}. This is again much lower than the sensitivity exhibited without anode control, but in keeping with the fact that the MFC-based biosensors were more sensitive to estradiol than BPA, with or without anode control.

Overall, these results suggest that control of anode potential is a crucial tool to help reduce variability in results by keeping the baseline performance of the MFCs constant, and their outputs very similar, *via* the means of overpotential. There are still some concerns with this conclusion – partly due to the low number of replicates, and some large error bars. Despite this major benefit they seem to provide, they do seem to significantly reduce sensitivity of the MFCs to these micropollutants under these conditions. This however, may be overcome by selection of a different overpotential, as demonstrated in some work by Stein *et al.* which shows the overpotential used can affect the proportion of the signal caused by a toxic event to the baseline. [13, 75] Longer-term exposure to control of anode potential has been shown to affect the structure of the biomass on the anode, and its function. Increasing the applied potential decreased biofilm thickness, which caused reduced charge transfer, and as a result, less substrate was oxidised. However, it was noted that biofilms has the highest potential did exhibit higher numbers of oxidases. Furthermore, the application of some

potential (0.3 V) was found to be better than no applied potential, implying the effect is not linear. [154] These effects could be responsible for the decreased sensitivity, and they reinforce the need for further work at a range of different potentials. Experiments using a longer-time period would also be useful in understanding if the results gained in these experiments change over time, or if any of the above changes in biofilm composition are exhibited over the course of the experiment. Additionally, the composition of species in the biofilm has been shown to be affected by longer-term exposure, which may affect the sensitivity of the MFCs to biologically active compounds. [155]

It has also been suggested that using "extremophiles", *i.e.* bacteria that thrive in extreme conditions, as part of the matrix of bacteria which form the biofilm may mean that higher anode potentials could be used, to harness greater overpotential, and enhance chemical oxidation. This could help to increase sensitivity, and would be an interesting area to explore further. [156] Use of such organisms would perhaps mean that a wider range of potentials could be investigated, without damage to the biofilm to help find the optimum.

In short, these results are promising, as they demonstrate how similar baselines and responses can be generated using this control method, but more work is needed to understand this effect fully. In particular, longer-term exposures to anode control would be useful in understanding some of their effects. However, these results also demonstrate this effect for the first time on non-metal micropollutants which is a big step forward.

5.5 – Removal of Micropollutants Using a Cascade of MFCs

As well as monitoring the concentration of emerging micropollutants and micropollutants in water streams, an important challenge to be faced is how to remove them in order to prevent potential damage to the environment and human health. [9] MFCs had first been suggested as a method of treating wastewater in 1991 by Habermann and Pommer, who developed MFCs which could treat wastewaters from sugar production and reduce the COD in the wastewater. They found that cells which reduced the COD most did not necessarily produce the most power due to differences in their design. [157] Since then, they have been seen as a potentially economically viable way to simultaneously treat wastewaters and generate electricity from a diverse range of sources such as potato farming and hospital effluent. [149]

In this study, during the course of micropollutant injections, it was noted that the concentration of the micropollutants decreased markedly, in part due to the metabolisation of the micropollutant, and in part possibly by absorption within the MFC. It was therefore decided to investigate if a more complete micropollutant removal could be achieved by passing the micropollutant through three MFCs, connected together in a cascade.

A cascade is defined as several MFCs hydraulically and possibly also electronically connected together with a sequential flow through the cells. Cascades have been shown previously to increase power output compared to individually-operated cells.[158]

The aim of these experiments is to help understand how much micropollutant can be removed by each cell, and if complete micropollutant removal could be achieved using multiple fuel cells. It would also demonstrate the electrochemical behaviour of each cell in series. This could demonstrate the suitability of MFCs for the treatment of wastewater in the context of removing complex biologically active molecules.

Each of the MFCs was fed clean feed independently for 12 hours. The feed to the first was then changed to include the micropollutant at 10 µg mL^{-1}, for 12 hours. A sample was taken at the end of this period, before the outlet of this MFC was fed into the next MFC for 12 hours, and so on, until all 3 cells were connected to the same source. A control experiment was run whereby feed without micropollutant was used throughout the same procedure. Furthermore, control cells being fed clean feed were also run while other experiments were in progress to help identify if any external factors were affecting the results. Each stage of the experimental setup is shown in Figure 65.

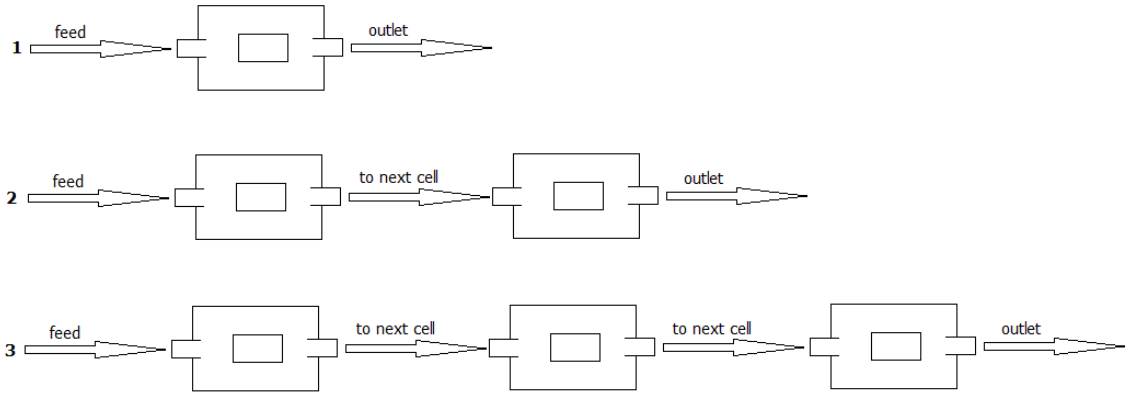

Figure 65 - Experimental Setup for Cascade Experiments

The samples collected at the outlet of each cell were analysed by LC-MS analysis to determine the reduction in concentration of each micropollutant caused by successive MFCs. Diclofenac was unable to be tested in this manner, due to a problem with its molecular mass being too similar to one of the components of the artificial wastewater. Triclosan was also not tested to allow our focus to be on the endocrine-disrupting chemicals, given limited instrument time.

In all cases, the traces below are formed by taking a reading every 10 seconds. The lines are point to point.

5.4.1 – Control Experiment

Results from a set of MFCs that were connected in a cascade, but without any micropollutant present are shown in Figure 66.

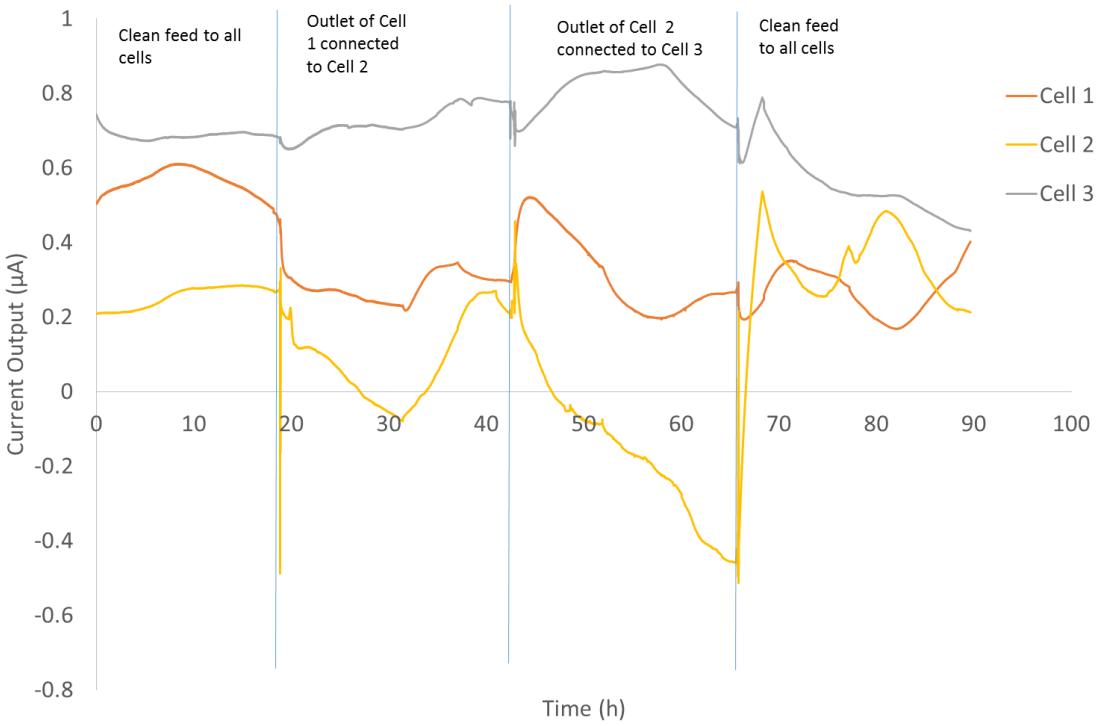

Figure 66 - Effect on the Output of 3 MFCs Fed with Clean Feed, Connected in a Cascade

These results show that when all cells were operating independently and fed feed without micropollutant, they provided relatively stable outputs over the time leading up to their connection in cascade. The connection of a second cell to Cell 8 decreased the performance of Cell 8 itself, and the performance of the cell which was connected, Cell 15. The connection of a third cell, however, seems to marginally improve the performance of the second and third cells, whilst decreasing the performance of the first cell. The first cell is driven into negative output when connected to other cells.

It is unclear exactly why the performance of the cells decreases with the connection of the second cell. This could be due to a reduction in flow rate, caused by extra resistance through the system. The improvement when the third cell is connected could be caused by the fact that the 2nd and 3rd cells are receiving partially metabolised substrate from the 1st cell, which is easier for them to metabolise and produce electrons with. The first cell could be negative as it is mostly not producing electrons – only partially metabolising the substrate. This effect has been demonstrated in a cascade of MFCs, whereby partially metabolised substrate from the first MFC in the sequence was passed to the next, and increased its performance. [159] The authors also demonstrated that this effect leads to increased power output of the cascade

as a whole, compared to the same number of individual cells. This work shows there is a metabolic interaction between the bacteria in different cells on some level, and could provide a tool to investigate these metabolic pathways and to optimise systems for the digestion of complex substrates.

When fed again with clean feed, the MFCs return to more normal performance, even though they remain in a cascade. However, they are somewhat more unstable in terms of output than prior to this experiment. This implies that any change to the biofilm or metabolic pathways of the bacteria is reversible to some extent, although some time is required until full performance is regained. Examples of such changes could include parts of the biofilm dying due to lack of nutrients caused by a reduced flow rate, or the activation of alternative metabolic pathways in the bacteria which would not be used to metabolise pure acetate.[69, 140]

5.4.2 – Estradiol

The output of the MFCs in the cascade experiments involving estradiol are found in Figure 67.

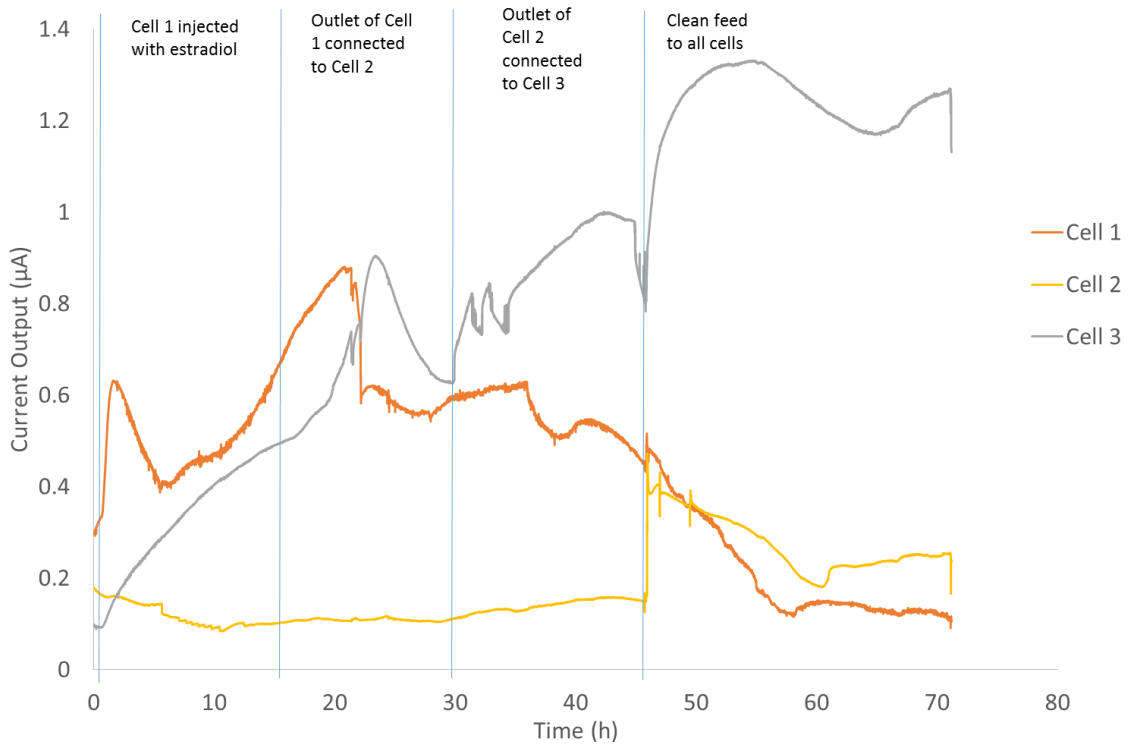

Figure 67 - Response of 3 MFCs to 10 µg mL^{-1} Estradiol, Connected in a Cascade

In general, the results show a trend that the injection of estradiol causes increased output of the MFCs. This seems to be true in all cases – at least initially. However, the first cell to be injected (Cell 15) eventually shows signs of decreasing output over time. This could be due to the long-term exposure to the estradiol – this trend was observed in some cells exposed to estradiol over long periods in other experiments. The increase in performance in cell 7 was much smaller than for the other cells, perhaps because the previous 2 cells had already oxidised all the available estradiol.

A return to clean feed in all cells provided an initial positive response, before the cells levelled out – perhaps this is due to the removal of the long term presence of estradiol which could have had a toxic effect on the cells.

Results from LCMS analysis of the outlets of each cell are shown in Figure 68.

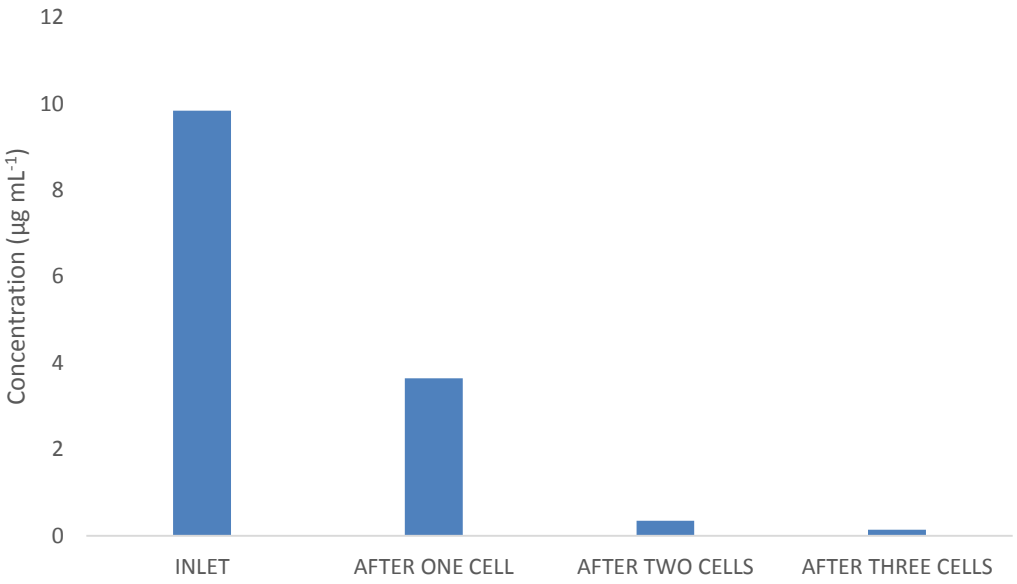

Figure 68 - Concentration of Estradiol After Each of the MFCs in a Cascade

The removal after one cell was 63.0%, after two cells was 96.4% and after all three cells, the removal was 98.5% of the original value. This is much greater than the 58.3% after one cell in the initial experiments, and shows that use of more than one MFC in a cascade can provide almost complete removal of this micropollutant. This is the first technology which can both sense and simultaneously remove the presence of such a biologically active micropollutant from a sample, which could have interesting implications for water treatment. Such a technology could prove highly valuable, as it can cheaply test and remove a micropollutant in a short space of time. However, more optimisation of the residence time/flow rate and the number of MFCs needed to provide complete removal is needed.

Since previous experiments highlighted the fact that some reduction of the concentration of the micropollutant was likely due to adsorption in the MFC, rather than metabolisation in the bacteria, it would also be beneficial to understand how the rate of adsorption varies in the MFCs, as it would have implications for the long-term use of an MFC cascade system to remove micropollutants.

5.4.3 – Bisphenol-A

The output of the MFCs in the cascade experiments involving bisphenol-a are found in Figure 69.

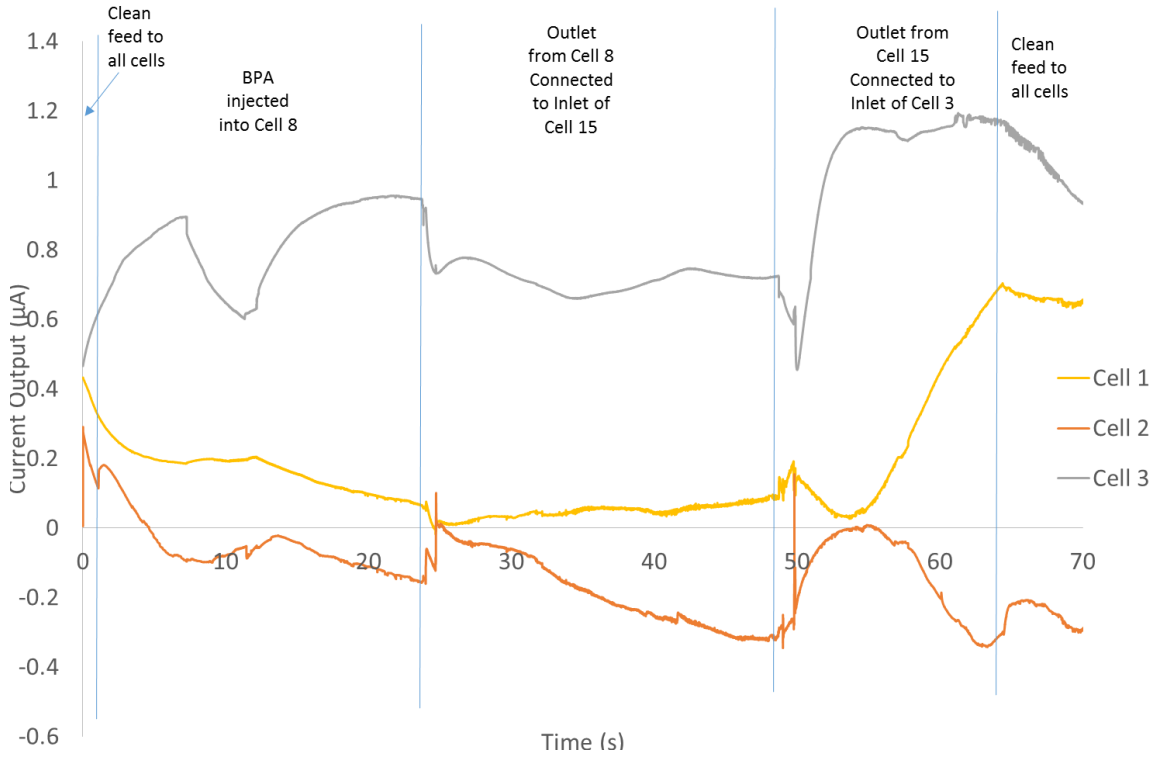

Figure 69 - Response of 3 MFCs to 10 µg mL^{-1} Bisphenol-A, Connected in a Cascade

The MFC injected with micropollutant first, Cell 8, initially had an increased output from the injection of bisphenol-a, but then its output decreased over time and eventually remained negative. Again this is in line with previous long term experiments from some cells. The connection of a second cell, Cell 15, caused a decreased output in that cell, and in Cell 8. Addition of the third cell, Cell 3 caused a rise in performance in all cells, at least initially. These results are very similar to those for estradiol, which implies that similar factors govern the output of the MFCs with micropollutants. This is unsurprising, considering the similarity in their results in other experiments and the similar nature of their toxicity.

Results from the LC-MS analysis of the outlets from each cell are shown in Figure 70.

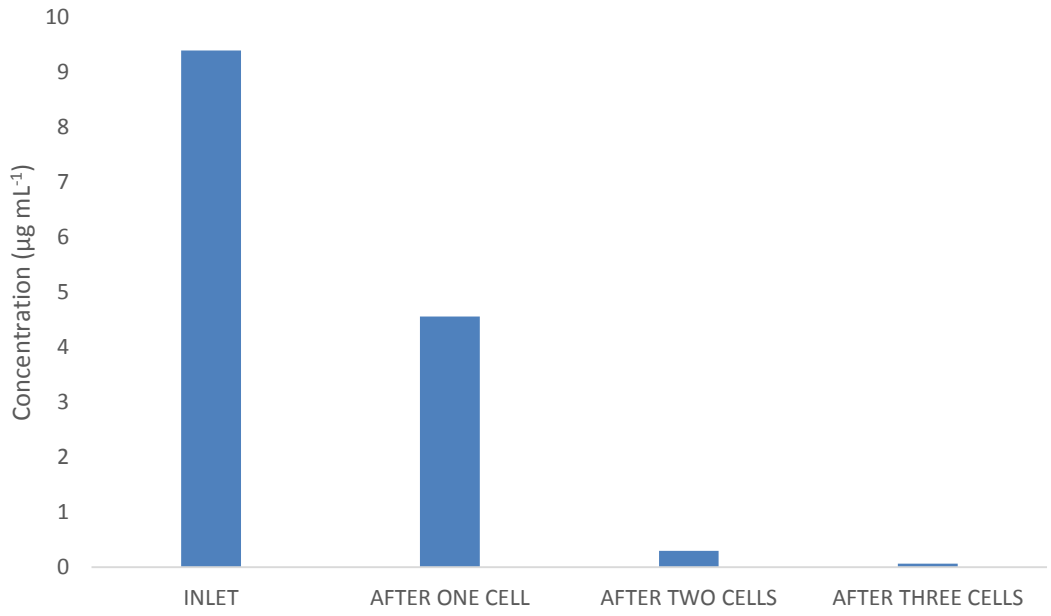

Figure 70 - Concentration of Bisphenol-A After Each of the MFCs in a Cascade

The removal after one cell was 51.5%, after two cells was 96.9% and after all three cells, the removal was 99.3% of the original value. Again, this is much greater than the value when the micropollutant was only passed through one cell for a shorter time. It implies that there is again, scope to apply this technology to both detect and remove the micropollutant simultaneously and that close to complete removal can be obtained.

These results are very promising, for both micropollutants tested. It would be interesting to apply these results to more micropollutants, and to optimise the process further to develop even better removal technologies. Research by others has shown that a cascade of seven MFCs can be used to reduce the COD of artificial wastewaters, using acetate as a feedstock. [158] They also reported an increase in power output of the cascade as a whole, compared to operating the cells individually, in line with other work. [159]

This demonstrates for the first time the use of such a technique to target the removal of a specific micropollutant, and uses a much smaller cascade to do so, which has cost advantages. Currently, this kind of organic micropollutant is not routinely specifically monitored in wastewater treatment processes, nor is it specifically targeted for removal; instead, it is removed using water treatment processes in place to remove generic organic materials, such as chemical oxidation, UV-treatment and others. [35] Work by several groups has highlighted that this kind of compound is not effectively treated by these measures[53, 56, 136, 160] and as such MFCs are a promising means by which this removal could be achieved.

Other research has indicted that bisphenol-a can be removed *via* the means of filtration through activated carbons.[161, 162] However, such processes are highly selective, based on the pore size of the activated carbon and may not be effective at removing every similar molecule. Furthermore, they operate within a certain pH range, which adds an extra complexity to the processing of the wastewater – MFCs can operate at neutral pHs, which would naturally be occurring in the water inlet.

Triclosan has been shown to be removed using enzymatic peroxidases, which are naturally occurring in organisms such as soybeans and horseradishes.[163] They break down the triclosan by metabolisation, in a similar manner to MFCs. However, since they are not a part of a wider organism, they lack the capacity to completely break down the triclosan, and it forms polymers during the metabolisation process, which must then be removed, requiring further treatments. MFCs may break down the triclosan more completely, and as such may not suffer this problem, but this would require study to confirm.

Estradiol has been found to be removed from wastewater in several ways – mostly through filtration means such as activated carbons, chitosan or ion exchange membranes. [164] Again, this process is selective by means of pore size, meaning MFCs have an advantage in that they can remove several types of micropollutants. Furthermore, the effectiveness of these methods was found to decrease in the presence of surfactants, even in low concentrations. Surfactants are commonly found in wastewater due to their ubiquitous use in the home, and this may present a problem in this case.

In summary, MFCs present a promising opportunity to remove similar organic compounds in a broad manner, as they have been shown to remove all three (although not simultaneously yet). Other technologies are selective to only one type of micropollutant due to their enzymatic nature or by nature of their pore size, giving MFCs a distinct advantage.

6.0 – CONCLUSIONS

This work outlines the potential for the use of MFCs as biosensors for biologically active organic micropollutants in water streams. They have been demonstrated to detect diclofenac, bisphenol-a, 17β-estradiol and triclosan. They were most sensitive to 17β-estradiol, perhaps owing to their well-known properties as endocrine disruptors, known to strongly affect the metabolisms of microorganisms. [57, 134, 135] The sensors exhibited a similar high sensitivity towards bisphenol-A, another endocrine disruptor [27, 28, 57], which helps reinforce this conclusion, whereas diclofenac, which is not known as a strong endocrine disruptor exhibited a much lower sensitivity. [137, 143]

It has been demonstrated that MFCs can act as biosensors for some of compounds in ranges close to the required levels found in wastewater. In wastewater, Bisphenol-A has been found between 0.088 and 11.8 ng L^{-1}. [135] Triclosan has been found in wastewater at levels between 3.8 and 16.6 µg L^{-1}.[136] Diclofenac has been found in the region of 119-1376 ng L^{-1}.[137]

Using these sensors, bisphenol-a and 17β-estradiol are clearly not detected at sufficiently low concentrations by these biosensors. The lower limit of detection was around a factor of 1,000 too high compared to their concentrations in wastewater, meaning this technology would require large advancements to become useful. These may not be forthcoming, or may take several years to develop.

However, triclosan was much closer – the lower detection limit was around the same as the highest concentration detected in wastewater in the study mentioned previously. This is promising as it demonstrates that with only a little enhancement, these biosensors could detect within a range useful for the treatment of wastewater. The sensors were also able to detecting diclofenac in close to sufficient concentrations, only being around a factor of 10 out from the range of concentrations found in that study.

This demonstrates that future research is needed in order to perfect these devices, but that they could be enhanced to become useful tools for the detection of some of the most important and potentially damaging emerging micropollutants. [6]

Whilst under control of external resistance, the biosensors suffered problems with stability. Often, the performance of different cells in terms of current output was not comparable, making comparing replicates difficult, and seeing underlying trends more challenging. However, as suggested by Stein *et al.*, controlling the MFCs *via* the means of anode potential

was shown to stabilise the baseline current of the cells, making them much more comparable and results were much more reproducible using this method. [13] This is potentially the best way forward to obtain reliable and quantifiable results , however to control the anode potential requires a potentiostat with an external power supply, which would limit the use of the MFCs *in-situ* as this places a requirement of laboratory conditions for them to operate.

The instability could arise from the fact that long-term exposure to biologically active compounds seems to have unexpected effects on the MFCs performance. This could be down to microbial resistance to the compounds injected over time [22] or due to physical changes in the biofilm that occur as a result of the metabolic changes caused by these compounds. [135]

There may be some long-term exposure effects which are as yet not understood, which are demonstrated by the fact that long-term exposure to one concentration of biologically active compound often seems to yield a different baseline current to the baseline current obtained before injection. They are, however, still suited to short shock-based testing for rapid, cheap detection of these compounds.

Any long term sensor would need to be reliable and consistent – results from different time frames would need to be comparable. [1, 63, 65] Unfortunately, these results do not provide that stability over time. However, sudden changes in output are noted when biologically active compounds are injected, meaning they are still suitable for short-term "shock" tests. [17]

In addition to acting as a biosensor, the MFCs have been demonstrated to provide almost complete removal of the compounds studied (except diclofenac, which could not be examined) over a range of concentrations. This is the first time that MFCs have been demonstrated to help remove organic micropollutants at such concentrations whilst simultaneously being able to detect their presence. This was achieved by using a sequential cascade, whereby the inlet was passed through several MFCs sequentially. Some removal seems to be through adsorption processes in the MFC (perhaps at the carbon cloth electrodes, or in the body of the MFC itself) and by metabolism of the analyte. Although MFCs have been shown to reduce the BOD or COD of wastewaters before, this is the first time they have been shown to reduce the levels of specific micropollutants of concern. [6, 37, 108]

However, these MFCs are obviously only small-scale devices with a 0.064 mL chamber volume. In order to be truly useful to treat wastewater, a much bigger volume of liquid would need to be handled. As discussed in the literature review, scaling up MFCs is difficult and can adversely affect their current output. It is unclear whether this would also adversely affect their rates of removal of micropollutants. The possibility to number up the MFCs also exists, whereby lots of micro-MFCs could operate side by side, to process the same volume of liquid as a larger MFC but maintaining the enhanced performance of the smaller devices. This would be the only possible way forward in this case, although a very large number of devices would be needed to cope with the volume of wastewater which flows through a water treatment plant, resulting in a very complex system.

Furthermore, it is unclear whether the same removal would be achieved in real wastewater, which is a much more complex medium and would comprise of a range of different potential fuel sources, in different concentrations, which may have all manner of different effects on the behaviour of the biofilm, or simply just be metabolised preferentially to the micropollutants under study.

In summary, this work has demonstrated that MFCs have potential to be used as biosensors for biologically active organic micropollutants, and remove them from artificial water streams.

The original objectives of this work were to:

1. To investigate the use of MFCs to detect diclofenac, triclosan, bisphenol-A and 17β-estradiol in an artificial wastewater medium
2. Investigate the potential long-term effects of these 4 micropollutants on the MFCs
3. Determine if these micropollutants can be removed from the inlet feed by metabolisation by the MFCs
4. Examine the use of external control of anode potential can help improve sensitivity towards these 4 compounds, and improve stability of the MFCs.

I believe this work has demonstrated that:

1. These four micropollutants have been shown to be detected by the MFCs in such a medium, at fairly low concentrations, some of which were close to the concentration typically found in real wastewater.

2. The long-term effects of exposure to micropollutants have been partially investigated, and seem to indicate that long-term exposure has an effect on the performance of the MFCs, although this varies by micropollutant.
3. That MFCs have shown significant potential to remove these micropollutants from an artificial wastewater feed, but that some of this removal appears to be due to absorption within the MFC, rather than metabolisation.
4. Controlling the external anode potential applied improved the sensitivity of the MFCs towards all of the micropollutants, and helped to stabilised the performance of the MFCs, to provide a more reliable baseline performance.

However more work is needed to:

- Understand the effects of long term exposure to these biologically active compounds upon the performance of the MFCs in general, and on their performance as biosensors
- Increase the stability of the current output of MFCs to maximise reproducibility and sensitivity
- Understand how the removal of these compounds from wastewaters is achieved
- Understand the physical processes affecting the biofilm
- Investigate whether multiple compounds could be detected simultaneously, or if a single compound could be detected in a complex medium

The possibilities for investigating these areas are explored in the following section.

7.0 – SUGGESTED FUTURE WORK

The most pressing issue facing these biosensors is their stability and reproducibility. Any sensor must be reliable and consistent – results taken at different times need to be comparable in order to establish whether or not an unacceptable concentration of any micropollutant has entered the system. [1, 63, 65] This is a basic requirement of any biosensor. The work undertaken to control the potential at the anode went some way to providing stable outputs and more reliable results. However, the potential chosen was based on work by Stein *et al.* and was based on a theoretical calculation. [13] It may be possible to find an overpotential which provides not only a stable current output and comparable results but also increases the sensitivity of the MFCs towards specific compounds. Such work has been carried out on metal based toxicants and has been found to enhance the sensitivity of MFCs towards various metals. [4, 75] However, no such work has been performed on the detection of organic compounds as yet.

Furthermore, it has been suggested that the use of bacteria which can tolerate more extreme conditions, and therefore higher electrical currents, might be beneficial in order to try a greater range of anode potentials in order to increase sensitivity of the MFCs. [156] This would work by providing the bacteria in the biofilm with extra energy in order to more completely oxidise the fuel source. It would be interesting to explore a wide range of potentials, in order to determine which would provide the optimum sensitivity.

Another interesting area to explore would be the makeup of the biofilm in general, and determine if different species are present depending on the length of exposure to the biologically active compounds, length of operation of the fuel cell and overpotential applied to the anode. This could be achieved by Denaturing Gradient Gel Electrophoresis of the biofilms at various stages in the process of biosensing. [165] This would reveal the species makeup of the biofilm and how it changes with environmental conditions. Work in this study highlighted physical changes in the biofilm with exposure to the biologically active compounds using SEM microscopy. It would be interesting to apply the same technique to biofilms exposed to different lengths of operation, and to different anode potentials as well in order to get a more complete picture of how biofilms are affected by their operating conditions.

Another area for investigation is the removal of the micropollutants from the feed source. It is important that any method of treating wastewater can work over an extended time period. The work in this study only covered timescales of a few days, but these systems

would need to be in continuous operation. [149] Since some of the micropollutant removal seemed to be attributed to absorption processes in the MFC rather than metabolisation by bacteria, as time passes this absorption will decrease as the capacity of the materials reduces with time. It would be interesting to take the MFC materials, soak them in a known quantity of solvent such as methanol, and run this methanol through LC-MS to quantify how much was absorbed by each component of the cell (body, electrodes, etc.). It would also be crucial to understand how much the removal rate of toxicants changes over longer timescales. Another interesting study would be to find out which molecules the organic molecules are metabolised into and look at their levels in the outlet. This could also be done by LC-MS as in the work by Petrie *et al.* and would provide insight as to whether metabolising these products genuinely reduces the environmental impact of their presence, or just releases similarly harmful compounds into the outlet. [130]

Obviously, real wastewater in the environment is a very complex medium, comprising of a whole manner of different organic molecules. The scope of this study was to monitor the concentration of a single biologically active organic micropollutant at once, in a simple artificial medium. This does not reflect real life applications of this technology, and a major challenge for the future will be the ability to detect compounds in a complex medium. Some work by Stein *et al.* has highlighted control of anode potential as a method for enhancing the sensitivity of MFCs towards a single toxic metal. [13] In theory, the signals of different toxicants might be enhanced by the use of different anode potentials, but this may not block out the signals of compounds with similar optimum potentials. The compounds in wastewater are so numerous that some are bound to have overlapping signals.

An alternative approach to this problem could be provided by a phenomena which has been observed several times – bacteria in MFCs seem to get used to metabolising a particular compound, and the correct metabolic pathways are activated in order to do so. It has been noted that when they are switched to an alternative fuel source, *e.g.* between acetate and glucose, there is a lag period where the bacteria do not metabolise so well. This lag is the delay between switching metabolic pathways. [106, 109, 112] In the case of the micropollutants in this study, which the MFCs seem to metabolise without negative effects, it might be possible to "prime" the MFCs by feeding them with a particular compound to be analysed for a preset period before introducing the medium to be analysed. They would perhaps more readily metabolise the compound under investigation than other carbon sources in the medium. Whether this effect would be prevalent enough to detect only a single compound in a complex medium is not yet clear.

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